Elucidation of Anti-tumor and Anti-inflammatory Constituents from *Melia azedarach* L.

（センダン由来抗腫瘍および抗炎症成分の探索）

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Chapter 1

Introduction

Melia azedarach L. (chinaberry tree; Meliaceae) is indigenous to Japan, Taiwan, China, and Southeast Asia. From ancient times, the bioactive plants have been used as folk medicine or Chinese medicine for treating patients. Bark and fruits of *M. azedarach* known as “Kurenpi” and “Kurenshi”, respectively, in Japan, have been used as the medicine for vermicide, anodyne, and skin disease. Various parts of *M. azedarach* have been traditionally used for insecticides, diuretics, astringent and stomachic as Indian traditional medicine in Ayurveda [1-3].

Limonoids are the modified terpenoids from apotirucallane affected by oxidation and elimination of four carbons. Plants of the Meliaceae family have been known as rich resource of limonoids. Limonoids have attracted much attention because of their structural diversity and broad range of bioactivities, such as antitumor, antimalarial, and antimicrobial [4]. Recently, it has become an attractive therapeutic target for the treatment of a number of diseases by natural limonoids.

According to previous phytochemical investigation, plants of the Meliaceae family have been well documented for their ability to metabolize structurally diverse and biologically significant limonoids and triterpenoids [5–9]. From various parts of *M. azedarach* many constituents including limonoids, triterpenoids, and steroids have been isolated [10-18]. Several of the limonoids isolated from *M. azedarach* have been reported to possess antifeedant [10, 12], and insecticidal activities [10], antimicrobial [11, 15], cytotoxic activities [16-17]. In those studies once, we can just know the structures of herbal medicine constituents including *M. azedarach*, but action mechanism for activities has not yet been clarified. Thus, the effectiveness of herbal

medicines and Chinese medicine can be clarified by accumulation of results concern with the biological activities and the action mechanism of natural medicine constituents. In addition, new material of herbal medicines origin for innovative drug developments can be offered.

In the present study, limonoids from the fruits, leaves and bark of *M. azedarach* were elucidated aiming to find bioactive constituents such as anti-tumor promoter and anti-tumor and anti-inflammatory agents. Namely, in order to confirm limonoids from *M. azedarach*, MeOH extract and hexane extract of fruits, MeOH extract of leaves and MeOH extract of bark were fractionated using any type of chromatography.

As the results, fifty-three compounds (**1**-**53**) including twenty new limonoids (**6**, **7**, **14**, **17**, **19**, **21**-**34**) were isolated from *M. azedarach*, and evaluation of their biological activities, such as inhibitory effects on induction of EVB-EA, cytotoxic activity and inhibitory activity against NO production were done.

From evaluation results of anti-tumor promotion, it was found that, thirty eight compounds (**2**-**9**, **16**, **17**, **19**-**35**, **37**, **40**, **41**, **44**, **46**-**53**) inhibited induction of Epstein-Bar virus early antigen (EBV-EA) activation induced by TPA in Raji cells [19].

Furthermore, it was clarified that compounds (**1**-**53**) were cytotoxic against cell lines of leukemia (HL60), lung (A549), duodenum (AZ521), breast (SK-BR-3). Among these compounds (**1**-**53**), three limonoids (**1**, **26**, and **33**), which exhibited potent cytotoxic activity, were further evaluated for their apoptosis inducing activity, and their mechanisms for apoptosis induction were analyzed by western blot [20-22].

In addition, it was disclosed that thirteen compounds (**10**, **16**, **25**, **26**, **29**, **31**-**34**, **42**, **44**-**46**) exhibited inhibitory activities against lipopolysaccharide (LPS)-induced nitric oxide (NO) release from macrophage (RAW 264.7) cell line with no, or almost no, cytoxicity. Furthermore, mechanism for potent inhibition of NO release by **16** was exhibited by western blot [23].

Chapter 2

Experimental Section

2.1 Plant Materials

Mature fruits of *M. azedarach* L. (Meliaceae) were collected from plants cultivated at the Toho University herbal garden (Funabashi-shi, Chiba, Japan) on 31 January, 2008. Leaves and bark of *M. azedarach* L. (Meliaceae) were collected from plants cultivated at the Toho University herbal garden (Funabashi-shi, Chiba, Japan) on 26 October, 2010. The plant material was authenticated by one (*K. K.*) of the authors, and a voucher specimen (TA-TH-MA-1001) was deposited in the herbarium of the School of Pharmaceutical Sciences, Toho University.

2.2 Spectroscopy and Determination of Physical Constants

Optical rotations were measured on a JASCO P-2010 polarimeter in EtOH or MeOH at 25℃. UV spect ra, on a JASCO V-630Bio spectrophotometer, and IR spectra, using a JASCO FTIR-300 E spectrometer, were recorded in EtOH and KBr disks, respectively. NMR spectra were recorded with a JEOL ECX-400 (^{1}H, 400 MHz; ^{13}C, 100 MHz) spectrometer in $CDCl_3$, C_5D_5N or $(CD_3)_2CO$. The chemical shifts (δ) values are given in ppm with TMS as internal standard, and coupling constants (*J*) in Hz. HRESIMS was recorded on an Agilent 1100 LC/MSD TOF (time-of-flight) system [ionization mode: positive; nebulizing gas (N_2) pressure: 35 psig; drying gas (N_2): flow, 12 L min^{-1}; temp: 325℃; capillary voltage: 3000 V; fragmentor voltage: 225 V]. Crystallizations were performed in MeOH, and melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected.

2.3 Chemicals and Reagents

Chemicals and reagents were purchased as follows: Fetal bovine serum (FBS; for RPMI-1640 medium), antibiotics (100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin), and non essential amino acid (NEAA) from Invitrogen Co. (Carlsbad, CA, U.S.A.), Dulbecco's modified Eagle's medium (D-MEM), Eagle's minimal essential medium (MEM), and thiazoyl blue tetrazolium bromide (MTT) from Sigma-Aldrich Japan Co. (Tokyo, Japan), 5-fluorouracil, the EBV cell-culture reagents, and n-butanoic acid from Nacalai Tesque, Inc. (Kyoto, Japan), and lipopolysaccharide (LPS) from Sigma-Aldrich Japan Co. (Tokyo, Japan); N^G-monomethyl-L-arginine (L-NMMA) from Calbiochem (San Diego, CA, USA); cisplatin and β–carotene from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and recombinant human (rh) Annexin V/FITC kit (Bender MedSystems) from Cosmo Bio Co. Ltd. (Tokyo, Japan). All other chemicals and reagents were of analysis grade.

2.4 Cell Lines and Culture Conditions

The cells were cultured according to the method previously reported [24-26]. Cell lines HL60 (leukemia), A549 (lung), SK-BR-3 (breast), and AZ521 (duodenum), and RPMI1788 (normal cell), were obtained from Riken Cell Bank (Tsukuba, Ibaraki), and RAW 264.7 murine macrophage cell line were obtained from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). Cell lines were cultured in the following media: HL60 and SK-BR-3 in RPMI-1640 medium, AZ521 and RAW 264.7 in DMEM medium, and A549 in 90% DMEM + 10% MEM + 0.1 mM NEAA. The medium was supplemented with 10% FBS and antibiotics (100 units/mL penicillin and 100 μg/m L streptomycin). Cells were incubated at 37 ℃ in a 5% CO_2 humidified incubator.

2.5 Chromatography

Preparative-TLC plates (20 x 20 cm) were coated with a 0.5 mm layer of Silica gel G60 (Merck & Co., Inc., Darmstadt, Germany). Silica gel (Silica gel 60, 230–400 mesh, Merck & Co. Inc) and C_{18} silica (ODS; Chromatorex-ODS, 100–200 mesh; Fuji Silysia Chemical, Ltd., Aichi) were used for open column chromatography. Reversed-phase preparative HPLC (with refractive index detector) was carried out on C_{18} silica columns (25 cm x 10 mm i.d.) at 25℃: on a Pegasil ODS-II 5 μm column (Senshu Scientific Co., Ltd.) with acetonitrile (MeCN) –H_2O–acetic acid (AcOH) (50:50:0.1; flow rate: 3.0 mL/min) (HPLC system I), MeCN –H_2O–AcOH (55:45:0.1; flow rate: 3.0 mL/min) (HPLC system II), and MeOH–H_2O–AcOH (35:65: 0.1; flow rate : 3.0 mL/min) (HPLC system III), MeOH–H_2O–AcOH (45:55: 0.1; flow rate : 3.0 mL/min) (HPLC system IV), MeOH–H_2O–AcOH (50:50:0.1; flow rate : 3.0 mL/min) (HPLC system V), MeOH–H_2O–AcOH (90:10:0.1; flow rate : 3.0 mL/min) (HPLC system VI); a Capcell Pak AQ column (Shiseido Co., Ltd., Tokyo) with acetonitrile (MeCN) –H_2O–acetic acid (AcOH) (48:52:0.1; flow rate: 3.0 mL/min) (HPLC system VII), MeCN –H_2O–AcOH (52:48:0.1; flow rate: 3.0 mL/min) (HPLC system VIII), MeCN –H_2O–AcOH (58:42:0.1; flow rate: 3.0 mL/min) (HPLC system IX), MeCN –H_2O–AcOH (60:40:0.1; flow rate: 2.0 mL/min) (HPLC system X), and MeOH–AcOH (40:60:0.1; flow rate : 2.0 mL/min) (HPLC system XI), MeOH–H_2O–AcOH (55:45:0.1; flow rate : 3.0 mL/min) (HPLC system XII), MeOH–H_2O–AcOH (68:32:0.1; flow rate : 2.0 mL/min) (HPLC system XIII); a TSK ODS-120T column (Toso Co., Ltd., Tokyo) with MeOH–H_2O–AcOH (68:32:0.1; flow rate : 3.0 mL/min) (HPLC system XIV).

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2.6 Extraction and Isolation

2.6.1 Isolation of Compounds from the MeOH Extract of *Melia azedarach* Fruits

The air-dried fruits of *M. azedarach* (19.7 kg) were pulverized and extracted with

hexane (reflux, 2 h, 3x), which gave a hexane extract (588 g). The defatted residue was, then, extracted with MeOH (room temp., 3 days, 3x) to give the extract (1036 g) which was partitioned in an EtOAc–H_2O mixture. The aq. layer was extracted with *n*-BuOH, and removal of the solvent under reduced pressure from the EtOAc-, *n*-BuOH-, and H_2O-soluble portions yielded 103 g, 260 g, and 658 g of the residue, respectively. A portion of the EtOAc fraction (91 g) was subjected to CC on a SiO_2 column (800 g). Step gradient elution was conducted with *n*-hexane–EtOAc (1:0 → 0:1) to give eleven fractions, A–K. Fraction F (3.07 g), from the eluate of *n*-hexane–EtOAc (3:2 → 1:1), was subjected to CC on a SiO_2 (120 g) using *n*-hexane–EtOAc (1:0 → 0:1) to give eight fractions, F1–F8. Preparative HPLC (system II) of fraction F5 (439 mg) yielded compound (**37**) (12.5 mg, t_R 22.4 min), compound (**35**) (64.0 mg, t_R 24.8 min), and compound (**38**) (2.2 mg, t_R 29.7 min). Fraction F6 (1.43 g) was subjected to an ODS CC [25 g; eluent: MeOH–H_2O (11:9 → 1:0)] to give four fractions, F6a–F6d. Prep. HPLC (system II) of fraction F6b (497 mg) yielded compound (**39**) (1.9 mg, t_R 29.3 min), compound (**40**) (1.5 mg, t_R 38.5 min), and compound (**15**) (1.2 mg, t_R 43.5 min). Fraction G (5.06 g), from the eluate of *n*-hexane–EtOAc (1:1 → 9:11), was subjected to CC on a SiO_2 (160 g) using $CHCl_3$–EtOAc (1:0 → 0:1) to give seven fractions, G1–G7. Upon CC on an ODS [70 g; MeOH–H_2O (1:1 → 0:1)] and subsequent CC on a SiO_2 [60 g; *n*-hexane–EtOAc (4:1 → 7:3)], fraction G3 yielded a fraction (74 mg) from which compounds (**12**) (2.6 mg, t_R 30.0 min) and compound (**18**) (7.4 mg, t_R 40.8 min) were isolated by HPLC (system XIV). Fraction G4 was passed through an ODS [50 g; MeCN–H_2O (2:3 → 1:0)] to give a fraction (206 mg) from which was isolated compound (**41**) (17.0 mg, t_R 42.0 min) by HPLC (system X). Fraction H (3.81 g), from the eluate of *n*-hexane–EtOAc (2:3), was subjected to CC on a SiO_2 [100 g; *n*-hexane–EtOAc (4:1 → 0:1)] to afford eleven fractions, Frs. H1–H11. Further CC on an ODS [30 g; MeOH–H_2O (9:1 → 3:2)] of fraction H7 (1.32 g) yielded six fractions, H7a–H7f. HPLC of fractions H7d (298 mg; system I) and H7f (146 mg; system XIII) yielded compound (**2**) (2.6 mg, t_R 30.0 min) and compound (**30**) (2.6 mg, t_R 30.0 min),

and compound (**31**) (2.6 mg, t_R 30.0 min) and compound (**20**) (2.6 mg, t_R 30.0 min). Preparative HPLC of fraction H8 (292 mg; system IX), eluted with *n*-hexane–EtOAc (11:9), gave compound (**1**) (4.2 mg, t_R 24.0 min), respectively.

2.6.2 Isolation of Compounds from the Hexane Extract of *Melia azedarach* Fruits

Air-dried fruits of *M. azedarachi* (19.7 kg) were pulverized and extracted with hexane (2 L/kg of dried fruits; 3h, reflux, 3×), and the residue was then extracted with MeOH [11]. The hexane extract (588 g) was subjected to CC (SiO_2 (500 g/40 g extract)) giving less-polar oily fraction (437 g) (hexane/EtOAc 1:0 to 4:1) and more-polar defatted fraction (52 g) (EtOAc/MeOH 1:0 to 0:1). The defatted fraction was applied to CC (SiO_2 (600 g); hexane/ EtOAc 1:0 to 0:1) giving nine fractions, Frs. A–I. Fr. D (2.82 g) from the eluate of hexane/EtOAc 11:9 was subjected to CC (SiO_2 (150 g); hexane/ EtOAc 9:1 to 0:1) to yield eight fractions, Frs. D1–D8. Further CC (ODS (13 g); MeOH/H_2O 2:3 to 1:0) and subsequent preparative HPLC (system XIV) of Fr. D2 (242 mg) afforded compound (**43**) (retention time (t_R) 63.0 min; 5.0 mg). Fr. D3 (1031 mg), upon CC (ODS (50 g); MeOH/H_2O 1:1 to 1:0) and subsequent preparative HPLC (system II), yielded compound (**35**) (t_R 32.8 min; 1.9 mg) and compound (**37)** (t_R 38.4 min; 2.1 mg). Fr. F (2.23 g) eluted with hexane/EtOAc 3:7 was applied to CC (SiO_2 (110 g); hexane/EtOAc 1:1 to 0:1) which yielded nine fractions, Frs. F1–F9. CC (ODS (27 g); MeOH/H_2O 2:3 to 1:0) of Fr. F4 (1038 mg) afforded seven fractions, Fr. F4-1–F4-7. Preparative HPLC (system VII) of Frs. F4-4 (318 mg) and F4-6 (188 mg) afforded compound (**29**) (t_R 27.2 min; 60.2 mg), compound (**31**) (t_R 24.0 min; 8.6 mg), and compound (**30**) (t_R 30.4 min; 11.9 mg), and compound (**18**) (t_R 52.0 min; 9.6 mg), respectively. Preparative HPLC (system I) of Fr. F6 (207 mg) yielded compound (**11**) (t_R 16.8 min; 7.0 mg) and compound (**36**) (t_R 20.0 min; 3.5 mg). Fr. G (2.53 g) eluted with hexane/EtOAc 1:4 was subjected to CC (ODS (57 g); MeOH/H_2O 2:3 to 1:0) which yielded ten fractions, Fr. G1–G10. Preparative HPLC of Frs. G3 (50 mg; system I), G5

(138 mg; system VIII), G7 (100 mg; system VIII), and G9 (35 mg; system I) yielded compound (**22**) (t_R 23.2 min; 1.8 mg), compounds compound (**13**) (t_R 32.0 min; 27.6 mg) and compound (**19**) (t_R 40.0 min; 21.1 mg), compound (**14**) (t_R 44.0 min; 3.0 mg) and compound (**16**) (t_R 56.0 min; 24.7 mg), and compound (**9**) (t_R 54.4 min; 1.9 mg), preparative Fr. H (2.27 g) from the eluate of hexane/EtOAc 1:4 was subjected to CC (SiO_2 (156 g); hexane/EtOAc 1:0 to 0:1) to yield five fractions, Frs. H1–H5. Further CC (ODS (57 g); MeOH/H_2O 4:5 to 1:0) and subsequent preparative HPLC (system VIII) of Fr. H3 (1437 mg) gave compound (**25**) (t_R 20.8 min; 6.3 mg) and compound (**21)** (t_R 28.0 min; 6.3 mg). Fr. I (1.33 g) eluted with hexane/EtOAc 1:9 was subjected to CC (ODS (40 g); MeOH/H_2O 2:3 to 1:0) to give nine fractions, Fr. I1–I9. Preparative HPLC of Frs. I6 (116 mg; system XIV) and I7 (99 mg; system I) yielded compound (**23**) (t_R 36.0 min; 3.0 mg) and compound (**24**) (t_R 31.2 min; 22.2 mg), and compound (**8**) (t_R 34.4 min; 15.4 mg), respectively.

2.6.3 Isolation of Compounds from *Melia azedarach* Leaves

The dried and powdered leaves of M. azedarach (698 g) were extracted with MeOH by maceration at r. t. (5L; 1 week, 3×) to yield a MeOH extract (76 g). The extract was suspended in H_2O and partitioned with EtOAC which yielded EtOAc- (29 g) and H_2O- (45 g) soluble fractions. To remove chlorophylls, the EtOAc-soluble fraction was subjected to CC (Diaion HP-20 (160 g)) and eluted with MeOH and acetone successively [48]. The almost chlorophyll-free MeOH eluate (21.1 g) was applied to CC (SiO_2 (800 g); hexane/EtOAc 9:1 to 0:1), which yielded thirteen fractions, Frs. 1–13. Fr. 4 (553 mg) from the eluate of hexane/EtOAc 7:3 was subjected to CC (Diaion HP-20 (40 g); MeOH/H_2O 4:1 and 1:0) to yield a fraction (34 mg). Preparative HPLC (system XII) of the fraction afforded compound (**45**) (retention time (t_R) 13.6 min; 2.1 mg). Fr. 5 (152 mg) from the eluate of hexane/EtOAc 7:3 was passed through CC (SiO_2 (15 g); hexane/EtOAc 3:2 to 1:4) to give a fraction (35 mg) which was subjected to preparative

HPLC (system XI) yielding compound (**44**) (t_R 11.2 min; 4.5 mg). Fr. 6 (298 mg) from the eluate of hexane/EtOAc 3:2 was applied to CC (ODS (6 g); MeOH/H_2O 1:1 to 9:1) to afford a fraction (118 mg), which, upon preparative HPLC (system V), afforded compound (**34**) (t_R 16.0 min; 5.8 mg). Fr. 8 (383 mg) from the eluate of hexane/EtOAc 2:3 was subjected to CC (ODS (11 g); MeOH/H_2O 1:1 to 0:1) to yield five fractions, Frs. 8a–8e. Crystallization of Fr. 8c (105 mg) from hexane/EtOAc was yielded compound (**29**) (73.7 mg). Fr. 9 (152 mg) from the eluate of hexane/EtOAc 3:7 was passed through CC (ODS (9 g); MeOH/H_2O 1:1 to 1:0) to yield six fractions, Frs. 9a–9e (35 mg). Preparative HPLC (system II) of Fr. 9d (30 mg) from the eluate of MeOH/H_2O 3:2 gave compound (**31**) (t_R 16.8 min; 2.9 mg). Fr. 9e (43 mg) from the eluate of MeOH/H_2O 7:3 was further applied to CC (SiO_2 (2 g); hexane/EtOAc 1:0 to 0:1), which yielded four fractions, Frs. 9e-1–9e-4. Preparative HPLC (system VIII) of Fr. 9e-3 (25 mg) from hexane/EtOAc afforded compound (**16**) (5.0 mg). Fr. 10 (215 mg) from the eluate of hexane/EtOAc 1:4 was applied to CC (SiO_2 (60 g); hexane/acetone 1:0 to 0:1) to yield six fractions, Frs. 10a–10f. Preparative HPLC (system VIII) of Fr. 10e (59 mg) yielded compounds (**25**) (t_R 16.0 min; 2.0 mg) and compound (**26**) (t_R 24.0 min; 1.5 mg). CC (ODS (8 g); MeOH/H_2O 3:2 to 1:0) of Fr. 11 (207 mg), from the eluate of hexane/EtOAc 1:9, yielded eight fractions, Frs. 11a–11f. Preparative HPLC (system V) of Frs. 11b (42 mg) and 11e (23 mg) afforded compound (**32**) (t_R 36.8 min; 2.2 mg) and compound (**10**) (t_R 16.0 min; 1.5 mg), respectively. Fr. 12 (383 mg) from the eluate of hexane/EtOAc 0:1 was subjected to CC (ODS (12 g); MeOH/H_2O 11:9 to 0:1) to yield seven fractions, Frs. 12a–12g. Preparative HPLC (system IV) of Fr. 12b (115 mg) yielded compound (**33**) (t_R 39.2 min; 3.1 mg). Fr. 13 (226 mg) from the eluate of hexane/EtOAc 0:1 was subjected to CC (ODS (12 g); MeOH/H_2O 11:9 to 0:1) to yield five fractions, Frs. 13a–13e. Fr. 13b (15 mg), which upon CC (SiO_2 (1 g); hexane/acetone 7:3 to 0:1), yielded compound (**46**) (3.7 mg) from the eluate of hexane/acetone 3:2.

2.6.4 Isolation of Compounds from *Melia azedarach* Bark

The dried and powdered bark of M. azedarach (2.8 kg) was extracted with MeOH by maceration at r. t. (7 l; 1 week, 3×) to yield a MeOH extract (162 g). The extract was suspended in H_2O and partitioned with EtOAc which yielded EtOAc- (84 g) and H_2O- (75 g) soluble fractions. The EtOAc-soluble fraction was applied to CC (SiO_2 (650 g); hexane/EtOAc 1:0 to 0:1), which yielded ten fractions, Frs. 1–10. Fr. 3 (7024 mg) from the eluate of hexane/EtOAc 4:1 was crystallized from EtOAc yielding compound (**47**) (73.0 mg). Fr. 5 (2293 mg) eluted with hexane/EtOAc 3:2 was subjected to CC (SiO_2 (115 g); hexane/$CHCl_3$ 1:0 to 0:1) to give eight fractions, Frs. 5a–5h. Preparative HPLC (system VII) of Fr. 5d (191 mg) from the eluate of hexane/CHCl3 7:3 yielded compound (**50**) (t_R 44.0 min; 3.3 mg) and compound (**49**) (t_R 73.6 min; 2.5 mg). CC (SiO_2 (80 g); hexane/EtOAc 1:0 to 0:1) of Fr. 8 (5048 mg), from the eluate of hexane/EtOAc 3:7, yielded four fractions, Frs. 8a–8d. Preparative HPLC (system VI) of Fr. 8d (235 mg) afforded compound (**42**) (t_R 28.1 min; 17.4 mg). Fr. 9 (6977 mg) from the eluate of hexane/EtOAc 1:4 was applied to CC (Diaion HP-20 (200 g); MeOH/H_2O 2:3 and 1:0) to yield five fractions, Frs. 9a–9e, of which Fr. 9b (882 mg), from the eluate of MeOH/H_2O 2:3, was a mixture of compounds (**52**, **53**), and Fr. 9d (5.5 mg), from the eluate of MeOH/H_2O 1:0, was consisted with a compound (**51**). Preparative HPLC (system III) of a portion of Fr. 9b (20 mg) yielded compound (**52**) (t_R 7.2 min; 13.0 mg) and compound (**53**) (t_R 11.2 min; 3.3 mg). Fr. 10 (12938 mg) from the eluate of hexane/EtOAc 0:1 was subjected to CC (Diaion HP-20 (320 g); MeOH/H_2O 2:3 and 1:0) to yield five fractions, Frs. 10a–10e. Further CC (SiO_2 (150 g); hexane/$CHCl_3$ 1:0 to 0:1) of Fr. 10d (3052 mg) from the eluate of MeOH/H_2O 1:0 yielded a fraction (76 mg), preparative HPLC (system XIII) of which gave compound (**48**) (t_R 48.0 min; 1.5 mg).

2.7 Bioassay

2.7.1 Inhibition of TPA Induced EBV-EA Activation

The EBV genome carrying human lymphoblastoid cells (Raji cells) were cultured in 10% fetal bovine serum (FBS) in RPMI-1640 medium (Sigma, St. Louis, MO). The indicator cells (Raji cells; 1×10^6 cells/mL) were incubated at 37 ℃ for 48 h in 1 mL of medium containing *n*-butyric acid (4 mM), TPA [32 pM = 20 ng in 2 μL dimethyl sulfoxide (DMSO)] and a known amount (32, 16, 3.2, 0.32 nmol) of the test compounds dissolved in 2 μL of DMSO. Smears were made from the cell suspension. The EBV-EA inducing cells were stained by the means of an indirect immunofluorescence technique. In each assay, at least 500 cells were counted, and the number of stained cells (positive cells) was recorded. The average EBV-EA induction of the test compound was expressed as a ratio relative to the control experiment (100%), which was carried out with *n*-butyric acid (4 mM) plus TPA (32 pM). EBV-EA induction was ordinarily around 40%. The viability of treated Raji cells was assayed by the Trypan blue staining method [27].

2.7.2 Cytotoxic Activity against Human Cancer Cell Lines

Cytotoxic activity assays was performed with HL60 (leukemia), A549 (lung), SK-BR-3 (breast), AZ521 (duodenum) (each 3×10^3 cell/well), and RAW264.7 (mouse macrophage)(1×10^4 cell/well) and RPMI1788 (normal cell line) were treated with test compounds for 48 h, and then MTT solution was added to each well. After incubation for 3 h, the blue formazan generated was solubilized with 0.04 M HCl in isopropanol. The absorbances at 570 nm (peak top) and 630 nm (bottom) were measured with a microplate reader (Tecan Japan Co., Ltd., Kawasaki). [26]

2.7.3 NO-Release Inhibition

RAW264.7 cells were plated at 1×10^5 cells/well in 96-well plates and treated with or without various concentrations of test samples (0.4 μL) for 2 h, followed by incubation with 1 μg/mL (100 μL) LPS for 16 h. Nitrite levels in culture media were determined using the *Griess* reagent and presumed to reflect NO levels [28]. Briefly, 100 μL of cell culture medium was mixed with 100 μL of *Griess* reagent (1% sulfanilamide, 0.1% naphthylethylene diamine in 2.5% H_3PO_4 soln.) and incubated at r.t. for 10 min. The absorbance was then measured at 570 nm using a microplate reader. Fresh culture media were used as blanks in all experiments.

2.7.4 Apoptosis Detection by Flow Cytometry

The rh Annexin V/FITC kit provides a rapid and convenient assay for apoptosis. The cells (HL60:1×10^5; AZ521:1.5×10^5) were exposed to test compounds. For flow cytometry, the cell samples prepared were washed with annexin-binding buffer and stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) for 10 min. The cell samples were analyzed by the flow cytometer (Cell Lab Quanta^{TM} SC) using the FL1 and FL2 ranges for annexin V–FITC and PI, respectively.[29,30]

2.7.5 Detection of Proteins by Western Blot

For isolation of whole-cell lysates, after incubation of the cells (HL60 and AZ521) at 37℃ for 24 hours, test compounds were added and incubated. Then, the cells were collected and lysed with extraction buffer. For isolation of cytosolic and mitochondria-rich fractions, the cells (AZ521) were collected and suspended in lysis buffer containing 0.05% digitonin. After the cell suspensions were incubated on ice for 20 min and centrifuged, the supernatants were saved as a cytosolic fraction, and the

pellets were further resuspended in the lysis buffer containing 1% NP-40. On ice for 20 min later, the supernatants were obtained as a mitochondria-rich fraction by centrifugation.

Lysates of total protein were separated by 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel-electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked later and incubated with either of anti-caspase-3, anti-caspase-8, and anti-AIF (Sigma-Aldrich Japan Co., Tokyo), and anti-cleaved caspase-3, anti-caspase-9, anti-β-actin, and anti-VDAC (Cell Signaling Technology, Beverly, MA) primary antibodies at 4 ℃ overnight. Then, the blots were detected with enhanced chemiluminescence (ECL) plus western blotting detection system (GE Healthcare, Chalfont, St. Giles, U.K.) [29-31].

Chapter 3

Structural Elucidation and Identification of Compouds

3.1 Structural Elucidation and Identification of Limonoids

3.1.1 Trichilin-type Limonoids

Seven trichilin type limonoids (**1**-**7**) including two new compounds (**6**, **7**) were isolated from the MeOH extract of *M. azedarach* fruits.

Among these compounds, five known compounds were identified as 12-*O*-acetylazedarachin B (**1**) [32], meliarachin C (**2**) [33], toosendanin (**3)**; C-29 epimeric mixture) [34], meliarachin K (**4**) [33], meliarachin G (**5**) [33] by HRMS, ^{1}H NMR, and ^{13}C NMR comparison with corresponding literature data (Figure 1).

The structures of two new compounds (**6**, **7**) were elucidated on the basis of spectroscopic data and by comparison with literature as described below, and their proposed structures were supported by analysis of the DEPT, ^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY data.

Compound (**6**) has the molecular formula $C_{29}H_{38}O_9$ (HRESIMS: *m/z* 553.2451 [M + Na]$^+$, $C_{29}H_{38}O_9Na^+$). The presence of three tertiary Me groups [δ_H 0.85 (s), 0.96 (s), and 1.04 (s)], an AcO group [δ_H 2.09 (s)], a MeO group [δ_H 3.31 (s)], a C-19/C-29 bridged acetal structure [δ_H 4.04 and 4.34 (each 1H, d, J = 12.3 Hz; H_2-19), and δ_H 4.16 (1H, s; H-29)], two keto C=O groups [δ_C 211.0 and 219.6], and β-substituted furyl group [δ_H 6.28, 7.29, and 7.39 (each 1H)] was revealed by the ^{1}H and ^{13}C NMR spectra (Tables 1 and 5). The above evidence, coupled with the IR absorptions at 3350 cm^{-1} (OH), 1732 and 1715 cm^{-1} (C=O), and 874 cm^{-1} (furan ring), led to the assumption that compound (**5**) was a trichilin-type of limonoid [32, 35]. The HMBCs from the *O*-bearing H-29 to

C-3, C-4, C-5, C-19, and MeO-29, and from the MeO-29 to C-29, combined with their shifts, located a MeO group at C-29. The *S*-configuration at C-29 (29-*endo*) was assigned from the chemical shift of H-3 [δ_H 4.92, (br. d), J = 2.8 Hz] which was compatible with that of the 29-*endo*-isomer of toosendanin (**3**) [δ_H 4.90, (br. d), J = 3.7 Hz] [32]. The 29-*exo*-isomer of compound (**3**) exhibited the H-3 signal at a low field [δ_H 5.37, (br. d), J = 3.7 Hz] [32]. The NOESY correlation (Figure 1) between H-3 and H-29 supported this assignment. The NMR spectra of compound (**6**) were very similar to those of compound (**4**) [33], except for the absence of an AcO group. The remaining AcO group was located at C-3 of compound (**6**) by the HMBC for H-3 and the AcO group [δ_C 169.8]. Hence, the structure of compound (**6**) was assigned as (29S)-19,29-epoxy-29-*O*-methyl-1α,3α,7α,29-tetrahydroxymeliacane-11,15-dione 3-acetate which was named 12-dehydroneoazedarachin D .

The molecular formula of compound (**7**) was determined as $C_{29}H_{38}O_9$ from its HRESIMS (*m/z* 553.2437 $[M + Na]^+$, $C_{29}H_{38}O_9Na^+$), same as that of compound (**6**). The ^1H and ^{13}C NMR spectra (Tables 1 and 5) of compound (**7**) resembled those of compound (**6**), except for the downfield shift of H-3 signal [δ_H 5.35, (br. d), J = 2.8 Hz] suggesting that compound (**7**) is a 29-*exo*-isomer [32] of compound (**6**). Compound (**7**) was assigned as (29*R*)-19,29-epoxy-29-*O*-methyl-1α,3α,7α,29-tetrahydroxymeliacane-11,15-dione 3-acetate (12-dehydro-29-*exo*-neoazedarachin D). The NOESY correlation (Figure 5) between H_2-19 and H-29 supported the configuration.

12-Dehydroneoazedarachin D [**6**]: Fine needles; mp 140–143 ℃; $[\alpha]_D^{25}$ +18.6 (*c* 0.51, MeOH); IR (KBr) ν_{max} cm^{-1}: 3350 (OH), 1732, 1715 (C=O), 874 (furan); ^1H NMR and ^{13}C NMR data are shown in Tables 1 and 5; HRESIMS *m/z*: 553.2451 $[M + Na]^+$ (calculated for $C_{29}H_{38}O_9Na$, 553.2413).

12-Dehydro-29-exo-neoazedarachin D [**7**]: Fine needles; mp 193–196 ℃; $[\alpha]_D^{25}$ –71.0 (*c* 0.48, EtOH); IR (KBr) ν_{max} cm^{-1}: 3368 (OH), 1730, 1710 (C=O), 874 (furan);

^{1}H NMR and ^{13}C NMR data are shown in Tables 1 and 5; HRESIMS *m/z*: 553.2437 [M + Na]$^{+}$ (calculated for $C_{29}H_{38}O_9Na$, 553.2413).

3.1.2 Vilasinin-type Limonoids

Three vilasinin-type limonoids (**8**-**10**) were isolated, including two compounds (**8**, **9**) from the MeOH extract of *M. azedarach* fruits and one compound (**10**) from the *M. azedarach* leaves. All of the three known compounds were identified as trichilinin D (**8**) [36], 1-*O*-cinnamoyltrichilinin (**9**) [37], trichilinin B (**10**) [37, 38], by HRMS, ^{1}H NMR, and ^{13}C NMR comparison with corresponding literature data (Figure 1).

3.1.3 Havanensin-type Limonoids

One havanensin-type limonoid (**11**) was isolated from the hexane extract of *M. azedarach* fruits. The known compound was identified as 3-deacetyl-1,6-diacetylsendanal (**11**) [39] by HRMS, ^{1}H NMR, and ^{13}C NMR comparison with corresponding literature data (Figure 1).

3.1.4 Salannin-type Limonoids

Twenty three salannin-type limonoids (**12-34**) including seventeen new limonoids (**14**, **17**, **19**, **21**-**34**) were isolated, including eighteen compounds (**12**, **13**, **15-28**, **30-31)** from the MeOH extract of *M. azedarach* fruits, thirteen compounds (**13**, **14**, **16**, **18**, **19**, **21**-**25**, **29**-**31)** from the hexane extract of *M. azedarach* fruits, and eight compounds (**16**, **25**, **27**, **29**, **31**-**34**) from *Melia azedrach* leaves.

Among these compounds, six known compounds were identified as salannin (**10**) [40], 3-*O*-deacetylsalannin (**11**) [41], 3-*O*-deacetyl-3-*O*- tigloylsalannin (**15**) [40], ohchinin (**16**) [42], ohchinin acetate (**18**) [43], 1-*O*-de- cinnamoyl-1-*O*-benzoylohchinin

acetate (**20**) [43] by HRMS, ^{1}H NMR, and ^{13}C NMR comparison with corresponding literature data (Figure 2).

The structures of seventeen new limonoids (**14**, **17**, **19**, **21**-**34**) were elucidated on the basis of spectroscopic data and by comparison with literature as described below, and their proposed structures were supported by analysis of the DEPT, ^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY data.

The molecular formula of compound (**14**) was determined as $C_{31}H_{40}O_8$ by its HRESIMS (*m/z* 563.2618 $[M + Na]^+$; $C_{31}H_{40}NaO_8^+$). The IR spectrum of compound (**14**) showed the absorption bands of OH (3367 cm^{-1}), ester C=O (1730 cm^{-1}), conjugated ester C=O (1713 cm^{-1}), and furyl (873 cm^{-1}) functions. The ^{13}C-NMR and HMBC data of compound (**14**) (Table 5) indicated the presence of 31 C-atoms including nine quaternary C-atoms (including four olefinic and two C=O C-atoms), eleven CH (including five O-bearing sp^3 and three olefinic ones), five CH_2 (including one O-bearing and one sp^2 ones), and six Me (including one O-bearing Me) groups. The ^{1}H- and ^{13}C-NMR data (Table 1 and Table 5) of compound (**14**) were closely similar to those of compound (**13**) [44], except for the presence of the 2-methyl-2-propenoyl (methacryl; Met) group [δ_H 2.05, s, Me(4′)], and [δ_H 5.65, t, J = 3.0 Hz; δ_H 6.16, *t*, J = 1.4 Hz; CH_2(3′)]; [δ_C 18.4, C(4′)], [δ_C 126.2, C(3′)]; [δ_C 136.2, C(2′)], and [δ_C 165.0, C(1′)] [23] instead of the tigloyl group at C(1) of compound (**13**). The HMBC cross-correlations of H–C(1) [δ_H 5.04, (d)] with C(1′) supported the presence of the methacryl group at C(1). In addition, the NOE interactions (Figure 5) between H–C(3), Me(29), Me(19), and H–C(1), and between Me(19), H–C(6), H–C(7), H_β–C(16), and H–C(17) supported the α-orientation of MetO-C(1), HO-C(3), and the furyl ring at C(17). The structure of compound (**14**) was elucidated as 1-methacrylsalannic acid methyl ester (= 3-deacetyl-4′-demethylsalannin).

Compound (**17**) displayed the molecular formula $C_{36}H_{42}O_8$ (HRESIMS: *m/z* 603.2945 $[M + H]^+$, $C_{36}H_{43}O_8^+$). The IR spectrum indicated the presence of OH (3367 cm^{-1}), C=O (1730 and 1710 cm^{-1}), cinnnamoyl (1640 and 1453 cm^{-1}) [45], and furan-ring (873 cm^{-1})

functions. The ^{1}H and ^{13}C NMR spectra (Tables 1 and 5) indicated the presence of three tertiary Me groups [δ_H 0.93, s; 1.19, s; and 1.31, s], a vinyl Me group [δ_H 1.65, d, J = 1.8 Hz], a COOMe group [δ_H 3.21, s; δ_C 172.8], and β-furyl group [δ_H 6.32, 7.26, and 7.31 (each 1H)]. In addition, the presence of a characteristic H_2-28 group [δ_H 3.60 and 4.05 (each d, J = 7.8 Hz)], forming a C-6 ether linkage as in ohchinin (**12**) [42], indicated compound (**17**) to be a salannin-type ring C-*seco*-limonoid. Compound (**17**) exhibited, moreover, the ^{13}C signals attributable to a cinnamoyl group (Table 5), and its ^{1}H and ^{13}C NMR spectra were almost superimposable on those of compound (**16**) [42], except for the coupling constant between the ^{1}H signals of H-2′ and H-3′. Compound (**16**) exhibited the signals with larger coupling constant between these signals ($J_{H\text{-}2',3'}$ = 15.9 Hz) [42] while compound (**17**) showed them with smaller coupling constant ($J_{H\text{-}2',3'}$ = 12.4 Hz) which implied that the latter was the *cis*-diastereoisomer of the former [46]. The structure of compound (**17**) was assigned as 1-*O*-*cis*-cinnamoylsalannic acid methyl ester (1-*O*-decinnamoyl-1-*O*-*cis*-cinnamoyl-ohchinin). The *cis*-cinnamoyl group of compound (**17**) was supported from the NOE correlation between the ^{1}H signals of H-2′ and H-3′ (Figure 5).

Compound (**19**) showed the molecular formula as $C_{34}H_{40}O_8$ (HRESIMS: *m/z* 599.2543 $[M + Na]^+$, $C_{34}H_{40}O_8Na^+$). The IR spectrum displayed absorptions ascribable to OH (3338 cm^{-1}), C=O (1735 and 1710 cm^{-1}), benzoyl (1657 cm^{-1}) [45], and furan-ring (872 cm^{-1}) functions. The ^{1}H and ^{13}C NMR data (Tables 1 and 5) of compound (**19**) were similar to those of compound (**17**), except for the presence of a benzoyl group and the absence of a *cis*-cinnamoyl group. In HMBC experiments, the H-1 signal at [δ_H 5.22, t, J = 3.2 Hz] showed a cross-peak with the carbonyl resonance [δ_C 165.1] of the benzoyl group, indicating that compound (**17**) had a benzoyl group at the C-1 position in place of a *cis*-cinnamoyl group in compound (**17**). The structure of compound (**19**) was elucidated as 1-*O*-benzoylsalannic acid methyl ester (1-*O*-decinnamoyl-1-*O*-benzoylohchininolide)**.**

Compound (**21**) was shown to have the molecular formula $C_{36}H_{42}O_9$ by HRESIMS

(*m/z* 641.2724 ([M + Na]$^+$, $C_{36}H_{42}O_9Na^+$). The IR spectrum displayed OH (3400 cm^{-1}), γ–lactone (1765 cm^{-1}) [47], C=O (1740 and 1717 cm^{-1}), and cinnamoyl (1637 and 1451 cm^{-1}) groups. The ^{1}H and ^{13}C NMR data (Tables 2 and 5) of compound (**21**) were analogous to those of compound (**16**) [42], although the β-furyl ring signals for compound (**16**) were lacking for compound (**21**). The presence of an α,β–unsaturated-γ-lactone ring instead of the β-furyl ring at C-17 was deduced by the ^{1}H [δ_H 4.27, 4.50 (each 1H, dt, *J* = 1.6, 18.0 Hz), H_2(23); and 7.06, dd, *J* = 1.2, 2.8 Hz; H(22)] and ^{13}C [δ_C 135.1, C(20); 174.3, C(21); 145.0, C(22); and 70.0, C(23)] signals [48]. The HMBC correlations for H-17 and the C-20, C-21, and C-22 indicated the presence of β-substituted-γ–lactone ring. Hence, the structure of compound (**21**) was assigned as 1-*O*-cinnamoyl-17-defurano-17-(2-buten-4-olide-2-yl)-salannic acid methyl ester which was named ohchininolide**.** The NOE correlation between H-17 and Me-18 supported that the γ-lactone ring at C-17 was α-oriented.

Compound (**22**) was revealed to have the molecular formula $C_{34}H_{40}O_9$ by HRESIMS (*m/z* 615.2560 [M + Na]$^+$, $C_{34}H_{40}O_9Na^+$). The IR spectrum displayed OH (3338 cm^{-1}), γ-lactone (1750 cm^{-1}), C=O (1732 and 1715 cm^{-1}), and benzoyl (1657 cm^{-1}) groups. The ^{1}H and ^{13}C NMR data (Tables 2 and 5) of compound (**22**) resembled those of compound (**21**), except for the presence of a benzoyl group and the absence of a cinnamoyl group. In HMBC experiments, the H-1 signal at [δ_H 5.18, t, *J* = 2.8 Hz] showed a cross-peak with the carbonyl resonance [δ_C 164.9] of the benzoyl group, indicating that compound (**22**) had a benzoyl group at C-1 position in place of a cinnamoyl group in compound (**21**). Hence, the structure of compound (**22**) was elucidated as 1-*O*-benzoyl-17-defurano -17-(2-buten-4-olide-2-yl)-salannic acid methyl ester (1-*O*-decinnamoyl-1-*O*-benzoyl-ohchininolide).

Compound (**23**) has been determined to have the molecular formula of $C_{37}H_{44}O_{10}$ at *m/z* 671.2821 ([M + Na]$^+$, $C_{37}H_{44}O_{10}Na^+$) in HRESIMS. The IR spectrum indicated the presence of OH (3361 cm^{-1}), γ-lactone (1766 cm^{-1}), C=O (1732 and 1712 cm^{-1}), and cinnamoyl (1639 cm^{-1}) groups. The ^{1}H and ^{13}C NMR data (Tables 2 and 5) of

compound (**23**) were closely related to those of compound (**21**), except for the presence of an additional MeO group. The MeO group was located to be at C-23 by the key HMBC for MeO-23 [δ_H 3.40 (s)] and C-23 [δ_C 102.6]. The relative configuration of compound (**23**) was mainly deduced from the NOESY spectrum, and also by comparing the NMR data with those of compound (**21**). Thus, compound (**23**) was determined to be (23ξ)-1-*O*-cinnamoyl-17-defurano-17-(4-methoxy-2-buten-4-olide-2-yl)-salannic acid methyl ester which was named 23-methoxyohchininolide A. The stereochemistry at C-23 of compound (**23**) remained undetermined.

Compound (**24)** was shown to have the molecular formula $C_{37}H_{44}O_{10}$ by HRESIMS (*m/z* 671.2835 $[M + Na]^+$, $C_{37}H_{44}O_{10}Na^+$), same as that of compound (**23**). The ^1H and ^{13}C NMR data (Tables 2 and 5), and the HMBC and NOESY data of compound (**24**) were similar to those of compound (**23**), with the differences being the downfield shift [*i.e.*, δ_H 5.57 (t, J = 1.2 Hz) compound (**24**) *vs.* 5.24 (s) compound (**23**)] of H-23 signal and upfield shift [*i.e.*, δ_H 3.21 (s) compound (**24**) *vs.* 3.40 (s) compound (**23**)] of MeO-23 signal in the ^1H NMR spectrum. This suggested that compound (**24**) was a stereoisomer at C-23 of compound (**23**), and the structure of compound (**24**) was assigned as (23ξ)-1-*O*-cinnamoyl-17-defurano-17-(4-methoxy-2-buten-4-olide-2-yl) -salannic acid methyl ester which was named 23-methoxyohchininolide B. The stereochemistry at C-23 of compound (**24**) remained undetermined.

The molecular formula of compound (**25**) was determined as $C_{36}H_{42}O_{10}$ from its HRESIMS (*m/z* 657.2666 $[M + Na]^+$, $C_{36}H_{42}O_{10}Na^+$). The IR spectrum indicated the presence of OH (3368 cm^{-1}), γ-lactone (1750 cm^{-1}), C=O (1735 and 1710 cm^{-1}), and cinnamoyl (1637 cm^{-1}) groups. The ^1H and ^{13}C NMR spectra (Tables 2 and 5) of compound (**25**) were similar to those of compound (**23**) and compound (**24**), except for the signals indicating an OH group at C-23 rather than a MeO group. The HMBC spectrum of compound (**25**) exhibited cross-peaks for H-22 [δ_H 6.11, t, J = 1.6 Hz] with C-17, C-20, C-21, and C-23, and H-23 [δ_H 5.46, (br. d), J = 9.6 Hz] with C-21 and C-22, which supported the presence of an OH group at C-23. Compound (**25**) was

characterized as (23ξ)-1-*O*-cinnamoyl-17-defurano-17-(4-hydroxy-2-buten-4-olide-2-yl)-salannic acid methyl ester (23-hydroxyohchininolide**).** The stereochemistry at C-23 of compound (**25**) remained undetermined.

Compound (**26**) was revealed to have the molecular formula $C_{34}H_{40}O_{10}$ by HRESIMS (*m/z* 631.2508 $[M + Na]^+$, $C_{34}H_{40}O_{10}Na^+$). The IR spectrum displayed OH (3368 cm^{-1}), γ-lactone (1750 cm^{-1}), C=O (1730 and 1717 cm^{-1}), and benzoyl (1655 cm^{-1}) groups. The ^1H and ^{13}C NMR data (Tables 3 and 6) of compound (**26**) resembled those of compound (**25**), except for the presence of a benzoyl group and the absence of a cinnamoyl group. In HMBC experiment, compound (**25**) exhibited cross peaks for H-1 [δ_H 5.28, t, J = 3.2 Hz] with C-3, C-5, and C-1′, indicating that compound (**26**) had a benzoyl group at the C-1 position in place of a cinnamoyl group in compound (**25**). The structure of compound (**26**) was elucidated as (23ξ)-1-*O*-benzoyl-17-defurano-17-(4-hydroxy-2-buten-4-olide-2-yl)-salannic acid methyl ester (1-*O*-decinnamoyl-1-*O*-benzoyl-23-hydroxyohchininolide). The stereochemistry at C-23 of compound (**26**) remained undetermined.

Compound (**27**) was shown to have the molecular formula $C_{36}H_{42}O_{10}$ by HRESIMS (*m/z* 657.2645 $[M + Na]^+$, $C_{36}H_{42}O_{10}Na^+$), same as that of compound (**21**). The IR spectrum exhibited the presence of OH (3350 cm^{-1}), γ-lactone (1760 cm^{-1}), C=O (1733 and 1710 cm^{-1}), and cinnamoyl (1638 cm^{-1}) groups. The ^1H and ^{13}C NMR spectra (Tables 3 and 6) of compound (**27**) were closely related to those of compound (**25**), although the 23-hydroxybut-20(22)-ene-21,23-γ-lactone ring signals for compound (**25**) were lacking for compound (**27**). Instead of the γ-lactone ring, the presence of an 21-hydroxybut-20(22)- ene-1,23- γ-lactone ring at C-17 was deduced by the ^1H [δ_H 5.81, s, H(21); 5.90, s, H(22)] and ^{13}C [δ_C 169.0, C(20); 97.7, C(21); 119.8, C(22); and 170.3, C(23)] signals [47, 49]. The HMBC cross-correlations for H-17 [δ_H 3.77, (br. d), J = 9.2 Hz] with C-20 and C-21, and H-22 [δ_H 5.90 (s)] with C-17, C-20, C-21, and C-23, and the NOE correlations between the ^1H signals of H-7β [δ_H 4.26, d, J = 3.2 Hz] and H-16β [δ_H 2.39 (*m*)], and H-16β and H-17β of compound (**27**) supported that the

γ-lactone ring was located at C-17 with an α-orientation. The structure of compound (**27**) was assigned as (21ξ)-1-*O*-cinnamoyl-17-defurano-17-(4-hydroxy-2-buten-4-olide-3-yl)-salannic acid methyl ester which was named 21-hydroxyisoohchininolide. The stereochemistry at C(21) of compound (**27**) remained undetermined.

The molecular formula of compound (**28**) was determined as $C_{32}H_{38}O_8$ from its HRESIMS (*m/z* 573.2523 $[M + Na]^+$, $C_{32}H_{38}O_8Na^+$). The IR spectrum indicated the presence of OH (3350 cm^{-1}), C=O (1733 and 1710 cm^{-1}), and cinnamoyl (1638 cm^{-1}) groups. The ^1H and ^{13}C NMR data (Tables 3 and 6) of compound (**28**) resembled those of compound (**16**) [42], except for the absence of the signals due to a furan-ring attached at C-17. Compound (**28**) exhibited, however, a ^{13}C signal due to a C=O group [δ_C 206.1] located most probably at C-17. The HMBC cross-correlations for H-16β [δ_H 2.85, dd, *J* = 3.2, 17.0 Hz] with C-14, C-15, and C-17, and Me-18 [δ_H 1.75, d, *J* = 1.8 Hz] with C-13, C-14, and C-17, confirmed the presence of a C=O group at C-17. The structure of compound (**28**) was assigned as 1-*O*-cinnamoyl-17-defurano-17-oxo-salannic acid methyl ester (17-defurano-17-oxoohchinin).

Compound (**29**) was indicated to have the molecular formula as $C_{32}H_{40}O_9$ by HRESIMS (*m/z* 569.2777 $[M + H]^+$, $C_{32}H_{41}O_9^+$). Its IR spectrum suggested the presence of hydroxy (3338 cm^{-1}), γ-lactone (1778 cm^{-1}), ester C=O (1731 cm^{-1}), conjugated ester C=O (1718 cm^{-1}), and furyl (860 cm^{-1}) functions. The ^{13}C-NMR data of compound (**29**) (Table 6), along with the DEPT data, indicated the presence of 32 C-atoms including ten quaternary C-atoms (including three C=O and two olefinic C-atoms), twelve CH (including five O-bearing sp^3 and four olefinic ones), three CH_2, and seven Me groups. The ^1H- and ^{13}C-NMR data (Table 3 and Table 6) of compound (**29**) were similar to those of compound (**4**), with obvious differences being the lack of oxygenated CH_2 group and the presence of C=O group [δ_c 177.5, C(28)] for compound (**29**) when compared with compound (**4**). This suggested the presence of a 6,28-γ-lactone ring in compound (**29**) like in nimbolide [50]. In the HMBC experiment, compound (**29**) exhibited cross peaks of H–C(5) [δ_H 2.39] and Me(29) [δ_H 1.25] with C(28) indicating

the presence of 6,28-γ-lactone ring structure in compound (**29**). Further, the NOE correlations (Figure 5) between H–C(1), Me(19), Me(29), and H–C(3), and between Me(19), H–C(6), Me(30), H–C(7), H_{β}–C(16), and H–C(17) supported the α-orientation of TigO-C(1), HO-C(3), the γ-lactone ring at C(6)/C(28), and the furyl ring at C(17). The structure of compound (**29**) was assigned as 3-deacetyl-28-oxosalannic acid methyl ester (= 3-deacetyl-28-oxosalannin).

Compound (**30**) was shown to have the molecular formula $C_{34}H_{38}O_9$ by HRESIMS (*m/z* 613.2412 $[M + Na]^+$, $C_{34}H_{38}O_9Na^+$). The IR spectrum indicated the presence of OH (3368 cm^{-1}), γ-lactone (1778 cm^{-1}), C=O (1730 and 1714 cm^{-1}), benzoyl (1656 cm^{-1}), and furan (873 cm^{-1}) groups. The ^1H and ^{13}C NMR data (Tables 3 and 6) of compound (**30**) were similar to those of compound (**19**), with obvious differences being the lack of oxygenated CH_2 group and the presence of C=O group [δ_C 177.6] at C-28 for compound (**30**) when compared with compound (**19**). This suggested the presence of a 6, 28-γ-lactone ring in compound (**30**) like in nimbolide [50]. In HMBC experiment, compound (**30**) exhibited cross peaks for H-5 [δ_H 3.56, d, *J* = 12.4 Hz] with C(3), C(4), C(6), C(7), C(10), C(19), and C(28), and [δ_H 1.29, *s*, Me(29)] with C(3), C(4), C(5), and C(28), supported the presence of 6,28-γ-lactone ring structure in compound (**30**), and the structure of compound (**30**) was assigned as 1-*O*-benzoyl-28-oxosalannic acid methyl ester (1-*O*-decinnamoyl-1-*O*-benzoyl-28-oxoohchinin).

Compound (**31**) showed a molecular formula $C_{31}H_{38}O_9$ as established by HRESIMS (*m/z* 577.2424 $[M + Na]^+$, $C_{31}H_{38}O_9Na^+$). The IR spectrum indicated the presence of OH (3338 cm^{-1}), γ-lactone (1772 cm^{-1}), C=O (1732 and 1714 cm^{-1}), and furan-ring (872 cm^{-1}) functions. The ^1H and ^{13}C NMR data (Tables 4 and 6) of compound (**31**) were analogous to those of compound (**30**), although the benzoyl signals for compound (**30**) were lacking for compound (**31**). The presence of the 2-methyl-2-propenoyl (methacryl) group instead of the benzoyl group at C-1 of compound (**30**) was deduced by the ^1H [δ_H 5.60, t, *J* = 1.6 Hz; and 6.22, (br. s), H_2(3′); and 2.03, s, Me(4′)] and ^{13}C [δ_C 166.2, C(1′); 136.3, C(2′); 126.0, C(3′), and 18.2, C(4′)] signals [51]. This was confirmed by

the HMBC correlations for H-1 [δ_H .4.87, t, *J* = 3.0 Hz] with C(3), C(10), and C(1′). The compound (**31**) was assigned as 1-*O*-methacryl-28-oxosalannic acid methyl ester (3-*O*-deacetyl-4′-demethyl-28- oxosalannin)**.**

Compound (**32**) showed the molecular formula $C_{32}H_{40}O_{10}$ by HRESIMS (*m/z* 607.2505 $[M + Na]^+$, $C_{32}H_{40}NaO_{10}^+$). The UV spectrum showed absorption maximum at 220 nm evidencing the presence of an α,β-unsaturated ketone system, while the IR spectrum showed the absorption bands of OH (3468 cm^{-1}), γ-lactone (1778 cm^{-1}), ester C=O (1748 cm^{-1}), and conjugated ester C=O (1709 cm^{-1}) functions. The ^{1}H- and ^{13}C-NMR spectroscopic data of compound (**32**) (Table 4 and Table 6) were analogous to those of compound (**29**) [21], although the furan-3-yl ring signals of compound (**29**) were lacking for compound (**32**). The presence of an α,β-unsaturated-γ-lactone ring instead of the furan-3-yl ring at C(17) was deduced from the ^{1}H-NMR [δ_H 4.77, (br. *s*), CH_2(23); and 7.28, *d*, *J* = 0.9 Hz, H–C(22)], and ^{13}C-NMR signals [δ_C 135.2, C(20); 174.1, C(21); 144.9, C(22); and 70.2, C(C23)] signals [23]. The HMBC correlations between H–C(17) [δ_H 3.60, (br. *d*), *J* = 8.7 Hz], and the C(20), C(21), and C(22) indicated that the β-substituted-γ-lactone ring as located at C(17). The structure of compound (**32**) was assigned as 3-deacetyl-28-oxosalannolactone. The NOE correlations between Me(30) [δ_H 1.36, *s*], Me(18) [δ_H 1.72, *d*, *J* = 1.4 Hz; H–C(17)]; and H_β–C(16) [δ_H 2.06–2.11 (*m*)] (Figure 5) supported the α-orientation of the γ-lactone ring at C(17).

Compound (**33**) had the molecular formula $C_{33}H_{40}O_{11}$ HRESIMS (*m/z* 623.2401[M + Na]$^+$, $C_{32}H_{40}NaO_{11}^+$). The UV absorption at 226 nm suggested the presence of an α,β-unsaturated ketone system, while the IR spectrum indicated the absorption bands of OH (3468 cm^{-1}), γ-lactone (1764 cm^{-1}), ester C=O (1741 cm^{-1}), and conjugated ester C=O (1709 cm^{-1}) functions. The ^{13}C-NMR and ^{1}H-NMR spectra of compound (**33**) (Table 4 and Table 6) resembled those of were closely similar to those of compound (**32**), although the α,β-unsaturated-γ-lactone ring signals for compound (**32**) were lacking for compound (**33**). Instead of the γ-lactone ring, the presence of a

21-hydroxybut-20(22)-ene-21,23-γ-lactone ring at C(17) was deduced from the ^{1}H-NMR [δ_H 5.80, (br. *s*); H–C(21); and 5.97, (br. *s*); H–C(22)], and ^{13}C-NMR signals [δ_c 168.8, C(20); 97.5, C(21); 120.4, C(22); and 169.8, C(23)] [20, 52]. The HMBC cross-correlations of H–C(17) [δ_H 3.85, (br. *d*), *J* = 9.2 Hz] with C(20), C(21), and C(22), and of H–C(22) with C(17) [δ_c 53.0], C(20), C(21), and C(23), and the NOE correlations between Me(30) [δ_H 1.34 (*s*)], Me(18) [δ_H 1.73, *d*, *J* = 1.4 Hz], and H–C(17) of compound (**33**) evidenced that the β–substituted-γ-lactone ring was located at C(17) with an α–orientation. Thus, the structure of compound (**33**) was elucidated as 3-deacetyl-28-oxoisosalanninolide. The configuration at C(21) of compound (**33**) remained undetermined.

The molecular formula of compound (**34**) was determined as $C_{28}H_{36}O_9$ on the basis of HRESIMS (*m/z* 539.2204 $[M + Na]^+$, $C_{28}H_{36}NaO_9^+$). The UV absorption at 223 nm suggested the presence of an α,β–unsaturated ketone system, while the IR spectrum indicated the absorption bands of OH (3494 cm^{-1}), γ-lactone (1784 cm^{-1}), ester C=O (1736 cm^{-1}), conjugated ester C=O (1712 cm^{-1}), and conjugated enone (1656 cm^{-1}) functions. The ^{1}H- and ^{13}C-NMR spectroscopic data of compound (**34**) (Table 4 and Table 6) resembled those of compound (**32**), except for the absence of the signals due to a furan-3-yl ring attached at C(17). Compound (**34**) exhibited, however, a ^{13}C signal due to a C=O group [δ_c 205.5] located most probably at C(17) [3]. The HMBC cross-correlations of H_β–C(16) [δ_H 2.87, *dd*, *J* = 6.4 Hz, 16.9 Hz] with [δ_c 134.5, C(13); 175.2, C(14); 79.1, C(15); and 205.5, C(17), and of Me(18) [δ_H 1.78, *d*, *J* = 1.8 Hz] with C(13), C(14), and C(17), confirmed the presence of a C=O at C(17). The structure of compound (**34**) was assigned as 3-deacetyl-17- defurano-17,28- dioxosalannin.

3-Deacetyl-4′-demethylsalannin (**14**): Amorphous solid. [α]D25 +49.1 (c = 0.57, EtOH). UV (EtOH): No absorption maximum above 210 nm. IR (KBr) 3367 (OH), 1730, 1713 (C=O); 873 (furan). ^{1}H NMR and ^{13}C NMR data are shown in Tables 1 and 5; HRESIMS *m/z*: 563.2618 ($[M + Na]^+$ (calculated for $C_{31}H_{40}NaO_8$, 563.2620).

1-*O*-Decinnamoyl-1-*O*-cis-cinnamoylohchinin (**17**): Fine needles; mp 131–134℃; $[\alpha]_D^{24}$ +50.7 (*c* 0.85, MeOH); UV (EtOH) λ_{max} (log ε) nm: 217 (3.61), 280 (3.41); IR (KBr) ν_{max} cm^{-1}: 3367 (OH), 1730, 1710 (C=O), 1640, 1453 (cinnnamoyl), 873 (furan); ^1H NMR and ^{13}C NMR data are shown in Tables 1 and 5; HRESIMS *m/z*: 603.2945 $[M + H]^+$ (calculated for $C_{36}H_{43}O_8$, 603.2957).

1-O-Decinnamoyl-1-O-benzoylohchinin (**19**): Fine needles; mp 155–158 ℃; $[\alpha]_D^{24}$ +62.2 (*c* 1.22, MeOH); UV (EtOH) λ_{max} (log ε) nm: 245 (3.78); IR (KBr) ν_{max} cm^{-1}: 3338 (OH), 1735, 1710 (C=O), 1657 (benzoyl), 872 (furan); ^1H NMR and ^{13}C NMR data are shown in Tables 1 and 5; HRESIMS *m/z*: 599.2543 $[M + Na]^+$ (calculated for $C_{34}H_{40}O_8Na$, 599.2620).

Ohchininolide (**21**): Fine needles; mp 134–137℃; $[\alpha]_D^{24}$ +103.5 (*c* 1.12, MeOH); UV (EtOH) λ_{max} (log ε) nm: 217 (4.36), 276 (4.13); IR (KBr) ν_{max} cm^{-1}: 3400 (OH), 1765 (γ-lactone), 1740, 1717 (C=O), 1638 (cinnamoyl); ^1H NMR and ^{13}C NMR data are shown in Tables 2 and 5; HRESIMS *m/z*: 641.2724 $[M + Na]^+$ (calculated for $C_{36}H_{42}O_9Na$, 641.2726).

1-*O*-Decinnamoyl-1-*O*-benzoylohchininolide (**22**): Amorphous solid; $[\alpha]_D^{25}$ +63.7 (*c* 0.88, EtOH); UV (EtOH) λ_{max} (log ε) nm: 226 (4.11); IR (KBr) ν_{max} cm^{-1}: 3338 (OH), 1750 (γ-lactone), 1732, 1715 (C=O), 1657 (benzoyl); ^1H NMR and ^{13}C NMR data are shown in Tables 2 and 5; HRESIMS *m/z*: 615.2560 $[M + Na]^+$ (calculated for $C_{34}H_{40}O_9Na$, 615.2570).

23-Methoxyohchininolide A (**23**): Fine needles; M.p. 136–139℃; $[\alpha]_D^{25}$ +160.2 (*c* 0.55, EtOH); UV (EtOH) λ_{max} (log ε) nm: 225 (4.20), 275 (4.06); IR (KBr) ν_{max} cm^{-1}: 3361 (OH), 1766 (γ-lactone), 1732, 1712 (C=O), 1639 (cinnamoyl); ^1H NMR and ^{13}C

NMR data are shown in Tables 2 and 5; HRESIMS *m/z*: 671.2821 $[M + Na]^+$ (calculated for $C_{37}H_{44}O_{10}Na$, 671.2832).

23-Methoxyohchininolide B (**24**): Fine needles; M.p. 132–135℃; $[\alpha]_D^{25}$ +141.0 (*c* 0.80, EtOH); UV (EtOH) λ_{max} (log ε) nm: 220 (4.08), 277 (3.96); IR (KBr) ν_{max} cm^{-1}: 3350 (OH), 1765 (γ-lactone), 1735, 1713 (C=O), 1638 (cinnamoyl); ^1H NMR and ^{13}C NMR data are shown in Tables 2 and 5; HRESIMS *m/z*: 671.2835 $[M + Na]^+$ (calculated for $C_{37}H_{44}O_{10}Na$, 671.2832).

23-Hydroxyohchininolide (**25**): Fine needles; M.p. 148–151℃; $[\alpha]_D^{24}$ +29.2 (*c* 1.30, EtOH); UV (EtOH) λ_{max} (log ε) nm: 217 (4.20), 276 (3.81); IR (KBr) ν_{max} cm^{-1}: 3368 (OH), 1750 (γ–lactone), 1735, 1710 (C=O), 1637 (cinnamoyl); ^1H NMR and ^{13}C NMR data are shown in Tables 2 and 5; HRESIMS *m/z*: 657.2666 $[M + Na]^+$ (calculated for $C_{36}H_{42}O_{10}Na$, 657.2675).

1-*O*-Decinnamoyl-1-*O*-benzoyl-23-hydroxyohchininolide (**26**): Amorphous solid; $[\alpha]_D^{25}$ +61.1 (*c* 1.48, EtOH); UV (EtOH) λ_{max} (log ε) nm: 226 (4.06), 274 (3.99); IR (KBr) ν_{max} cm^{-1}: 3368 (OH), 1750 (γ-lactone), 1730, 1717 (C=O), 1655 (benzoyl); ^1H NMR and ^{13}C NMR data are shown in Tables 3 and 6; HRESIMS *m/z*: 631.2508 $[M + Na]^+$ (calculated for $C_{34}H_{40}O_{10}Na$, 631.2519).

21-Hydroxyisoohchininolide (**27**): Fine needles; M.p. 150–153℃; $[\alpha]_D^{25}$ +327.1 (*c* 0.30, EtOH); UV (EtOH) λ_{max} (log ε) nm: 220 (4.11), 276 (3.90); IR (KBr) ν_{max} cm^{-1}: 3350 (OH), 1760 (γ-lactone), 1733, 1710 (C=O), 1638 (cinnamoyl); ^1H NMR and ^{13}C NMR data are shown in Tables 3 and 6; HRESIMS *m/z*: 657.2645 $[M + Na]^+$ (calculated for $C_{36}H_{42}O_{10}Na$, 657.2675).

17-Defurano-17-oxoohchinin (**28**): Fine needles; M.p. 125–128℃; $[\alpha]_D^{25}$ +92.5 (*c* 0.54, EtOH); UV (EtOH) λ_{max} (log ε) nm: 217 (4.34), 240 (4.10), 279 (4.41); IR (KBr)

ν_{max} cm^{-1}: 3350 (OH), 1733, 1710 (C=O), 1638 (cinnamoyl); ^{1}H NMR and ^{13}C NMR data are shown in Tables 3 and 6; HRESIMS *m/z*: 573.2523 $[M + Na]^+$ (calculated for $C_{32}H_{38}O_8Na$, 573.2464).

3-Deacetyl-28-oxosalannin (**29**): Fine needles; M.p. 200–203℃; $[\alpha]_D^{24}$ +50.7 (c = 0.85, MeOH). UV (EtOH): No absorption maximum above 210 nm. IR (KBr) 3338 (OH), 1778 (γ-lactone) 1731, 1718 (C=O), 860 (furan); ^{1}H NMR and ^{13}C NMR data are shown in Tables 3 and 6; HRESIMS *m/z*: 569.2777 $[M + H]^+$ (calculated for $C_{32}H_{41}O_9$, 569.2750).

1-*O*-Decinnamoyl-1-*O*-benzoyl-28-oxoohchinin (**30**): Fine needles; M.p. 204–207 ℃; $[\alpha]_D^{25}$ +123.8 (*c* 0.76, EtOH); UV (EtOH) λ_{max} (log ε) nm: 230 (3.91); IR (KBr) ν_{max} cm^{-1}: 3368 (OH), 1778 (γ-lactone) 1730, 1714 (C=O), 1656 (benzoyl), 873 (furan); ^{1}H NMR and ^{13}C NMR data are shown in Tables 3 and 6; HRESIMS *m/z*: 613.2412 $[M + Na]^+$ (calculated for $C_{34}H_{38}O_9Na$, 613.2413).

3-*O*-Deacetyl-4′-demethyl-28-oxosalannin (**31**): Fine needles; M.p. 228–231℃; $[\alpha]_D^{25}$ +87.4 (*c* 0.19, EtOH); IR (KBr) ν_{max} cm^{-1}: 3338 (OH), 1772 (γ-lactone), 1732, 1714 (C=O), 872 (furan); ^{1}H NMR and ^{13}C NMR data are shown in Tables 4 and 6; HRESIMS *m/z*: 577.2424 $[M + Na]^+$ (calculated for $C_{31}H_{38}O_9Na$, 577.2413).

3-Deacetyl-28-oxosalannolactone (**32**): Fine needles (MeOH). M.p. 131–134 °. $[\alpha]^{25}{}_D$: +65.5 (*c* = 0.44, EtOH). UV (EtOH): 220 (3.92), 284 (3.58). IR (KBr): 3468 (OH), 1778 (γ-lactone), 1749 (ester C=O), 1709 (conjugated ester C=O); ^{1}H NMR and ^{13}C NMR data are shown in Tables 4 and 6; HRESIMS *m/z*: 607.2505 $[M + Na]^+$(calculated for $C_{32}H_{40}NaO_{10}$, 607.2519).

3-Deacetyl-28-oxosalanninolide (**33**): Fine needles (MeOH). M.p.162–165 °. $[\alpha]^{25}{}_D$:

+82.7(*c* = 0.75, EtOH). UV (EtOH): 226 (3.95), 286 (3.63). IR (KBr): 3468 (OH), 1764 (γ-lactone), 1741 (ester C=O), 1709 (conjugated ester C=O); ^{1}H NMR and ^{13}C NMR data are shown in Tables 4 and 6; HRESIMS *m/z*: 623.2401 [*M* + Na]$^{+}$(calculated for $C_{32}H_{40}NaO_{11}$, 623.2468).

3-Deacetyl-17-defurano-17,28-dioxosalannin (**34**): Fine needles (MeOH). M.p. 115–118º[α]$^{20}_{D}$: +6.0 (*c* = 1.31, EtOH). UV (EtOH): 223 (3.91). IR (KBr): 3494 (OH), 1784 (γ-lactone), 1736 (ester C=O), 1712 (conjugated ester C=O), 1656 (conjugated enone); ^{1}H NMR and ^{13}C NMR data are shown in Tables 4 and 6; HRESIMS *m/z*: 539.2204 [*M* + Na]$^{+}$(calculated for $C_{28}H_{36}NaO_{9}$, 539.2257).

3.1.5 Nimbin-type Limonoids

Five nimbin-type limonoids (**35-39**) including one new compound (**36**) were obtained from *M. azedarach* fruits, while compounds (**35**, **37-39**) were isolated from the MeOH extract and compounds (**35-37**) were isolated from the hexane extract.

Among these compounds, four known compounds (**35**, **37-39**) were identified as ohchinolal (**35**) [42], mesendanin E (**37**) [51], 1-*O*-detigloyl-1-*O*-benzoylohchinolal (**38**) [45], 1-*O*-detigloyl-1-*O*-cinnamoyl-ohchinolal (**39**) [45], by HRMS, ^{1}H NMR, and ^{13}C NMR comparison with corresponding literature data (Figure 3).

The structure of one new compound (**36**) was elucidated on the basis of spectroscopic data and by comparison with literature as described below, and their proposed structures were supported by analysis of the DEPT, ^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY data.

Compound (**36**) gave the molecular formula as $C_{29}H_{38}O_{8}$ (HRESIMS: *m/z* 531.2579 [M + H]$^{+}$, $C_{29}H_{39}O_{9}^{+}$). The IR spectrum of compound (**36**) indicated the presence of OH (3360 cm^{-1}), ester C=O (1740 cm^{-1}), and furyl (870 cm^{-1}) functions. The ^{13}C-NMR data of compound (**36**) (Table 6) indicated the presence of 29 C-atoms including eight

quaternary C-atoms (including two C=O and three olefinic C-atoms), twelve CH (including five O-bearing sp^3, one O-bearing sp^2, and three olefinic ones), three CH_2, and six Me groups. The ^{1}H- and ^{13}C-NMR data (Table 6) of compound (**36**) were analogous to those of compound (**35**) [42], although the signals of the tigloyl group of ohchinolal were lacking for compound (**36**), suggesting that it possesses the structure of 1-detigloylohchinolal. The NOE interactions (Figure 5) between H–C(3), Me(29), Me(19), and H–C(1), between Me(19), H–C(6), H–C(7), H_β–C(16), and H–C(17), and between Me(19) and Me(30) supported the relative configurations proposed.

1-Detigloylohchinolal (**36**): Fine needles. M.p.129–132ºC. $[\alpha]_D^{24}$ +84.5 (*c* = 1.01, MeOH). UV (EtOH): 220 (4.17). IR (KBr) 3360 (OH), 1740 (C=O), 870 (furan); ^{1}H NMR and ^{13}C NMR data were showed as Tables 4 and 6; HRESIMS: *m/z* 531.2579 [M + H]$^+$ (called for $C_{29}H_{39}O_9$, 531.2594).

3.1.6 Nimbolinin-type Limonoids

One nimbolinin-type Limonoids (**40**) was isolated from the MeOH extract of *M. azedarach* fruits. The known compound was identified as nimbolinin D (**40**) [53] by HRMS, ^{1}H NMR, and ^{13}C NMR comparison with corresponding literature data (Figure 3).

3.2 Structural Elucidation and Identification of Other Compounds

Three triterpenoids (**41**-**43**), one apocarotenoind (**44**), one alkaloid (**45**) seven steroids (**46**-**51**) and one flavonoid (**52**) were obtained. As these compounds, two compounds (**41**, **43**) were isolated from the *M. azedarach* fruits, three compounds (**44**, **45**, **46**) were isolated from the *M. azedarach* leaves, and seven compounds (**42**, **47**-**52**) were isolated from the *M. azedarach* bark;

All these known compounds were identified as meliasenin E (**41**) [54], methyl kulonate (**42**) (= methyl 3-oxo-16β-hydroxyeupha-7,24-dien-21-oate) [55] , azedarachic acid (**43**) [56], loliolide (**44**) [57], methyl indole 3-carboxylate (**45**) [58], 2α,3α,20*R*-trihydroxy-5α-pregnane 16β-methacrylate (**46**) [59], sitosterol (**47**) [60], 2,19-oxymeliavosin (**48**) [61], toosendansterol A (=3β,20*S*-dihydroxy-5α-pregnan-16-one; **49**) [62], 5,6-dehydrotoosendansterol A (=(20ξ)-3β,20-dihydroxypregn-5-en-16-one; **50**) [63], sitosterol 3-*O*-β-D-glucopyranoside (**51**) [64], (+)-catechin (**52**) [65], and (–)-epicatechin (**53**) [65] by HRMS, ^{1}H NMR, and ^{13}C NMR comparison with corresponding literature data (Figure 3 and 4).

Methyl kulonate (**42**): ^{1}H-NMR (400 MHz, $CDCl_3$): 0.83 (*s*, Me(18)); 1.02 (*s*, Me(19)); 1.04 (*s*, Me(29)); 1.12 (*s*, Me(28)); 1.27 (*s*, Me(30)); 1.46 (*dd*-like, *J* = 4.1, 14.7, H_α–C(1)); 1.58 (*s*, Me(27)); 1.58–1.67 (*m*, CH_2(11), H_a–C(12), H_α–C(15), and H_α–C(22)); 1.68–1.73 (*m*, H–C(5)); 1.69 (s, Me(26)); 1.94–2.04 (*m*, H_β–C(1), H_β–C(12), H_β–C(22), and CH_2(23)); 2.05–2.11 (*m*, H_β–C(15) and H–C(17)); 2.07–2.13 (*m*, CH_2(6)); 2.25 (*dt*, *J* = 3.9, 14.2, H_a–C(2)); 2.26–2.32 (*m*, H–C(9)); 2.60 (*dt*, *J* = 4.1, 10.5, H–C(20)); 2.77 (*dt*, *J* = 5.5, 14.7, H_β–C(2)); 3.72 (s, 21-MeO); 3.98 (*dd*, *J* = 5.9, 7.8, H–C(16)); 5.06 (br. *t*, *J* = 7.0, H–C(24)); 5.31 (*dd*, *J* = 2.8, 6.2, H–C(7)). ^{13}C-NMR (100 MHz; $CDCl_3$): 12.7 (C(19)); 17.6 (C(27)); 17.9 (C(11)); 21.5 (C(28)); 23.5 (C(18)); 24.3 (C(6)); 24.5 (C(29)); 25.7 (C(26)); 25.8 (C(23)); 27.8 (C(30)); 31.3 (C(22)); 33.0 (C(12)); 34.8 (C(2)); 35.0 (C(10)); 38.4 (C(1)); 44.6 (C(15)); 45.5 (C(13)); 47.2 (C(20)); 47.8 (C(4)); 47.9 (C(9)); 49.8 (C(14)); 51.7 (21-MeO); 52.3 (C(5)); 58.6 (C(17)); 77.2 (C(16)); 118.5 (C(7)); 123.3 (C(24)); 132.4 (C(25)); 144.6 (C(8)); 177.7 (C(21)); 216.8 (C(3)). The NMR signal assignment was performed by 2D NMR spectroscopic analysis and by comparison of the spectral data with those of meliasenin E (= methyl 3-oxo-16β,29-dihydroxyeupha-7,24-dien-21-oate) [54].

5,6-Dehydrotoosendanesterol A (**50**): ^{1}H-NMR (400 MHz, $CDCl_3$): 0.95 (*s*, Me(18));

1.05 (*s*, Me(19)); 106–1.12 (*m*, H_{α}–C(1)); 1.13 (*dt*, *J* = 6.4, 16.4, H–C(9)); 1.42 (*d*, *J* = 6.4, Me(21)); 1.45–1.55 (*m*, H_{α}–C(7)), H_{α}–C(11)), H_{α}–C(12)), and H–C(14)); 1.56–1.68 (*m*, H–C(8) and H_{β}–C(11)); 1.62–1.69 (*m*, H_{α}–C(2)); 1.83–1.90 (*m*, H_{β}–C(1), H_{β}–C(2), and H_{α}–C(15)); 1.92 (*d*, *J* = 6.4, H–C(17)); 1.93–2.00 (*m*, H_{β}–C(7)); 2.23–2.30 (*m*, H_{β}–C(12)); 2.25 (*dd*, *J* = 7.3, 11.4, H_{α}–C(4)); 2.26–2.31 (*m*, H_{β}–C(15)); 2.32 (*ddd*, *J* = 2.0, 5.6, 12.0, H_{β}–C(4)); 3.54 (*tt*, *J* = 5.6, 11.0, H–C(3)); 4.08 (*dq*, *J* = 6.4, 7.4, H–C(20)); 5.36 (*d*-like, *J* = 5.5, H–C(6)). ^{13}C-NMR (100 MHz; $CDCl_3$): 13.7 (C(18)); 19.5 (C(19)); 20.6 (C(11)); 23.2 (C(21)); 30.7 (C(8)); 31.6 (C(7)): 31.9 (C(2)); 36.7 (C(10)); 37.0 (C(1)); 39.2 (C(15)); 39.3 (C(12)); 42.3 (C(4)); 42.7 (C(13)); 50.0 (C(9)); 50.7 (C(14)); 66.6 (C(20)); 69.5 (C(17)); 71.7 (C(3)); 121.0 (C(6)); 141.1 (C(5)); 217.8 (C(16)). The NMR signal assignment was performed by 2D NMR spectroscopic analysis and by comparison of the spectral data with those of compound (**46**) [62] and cholesterol [60]. The stereochemistry at C(20) remained undetermined.

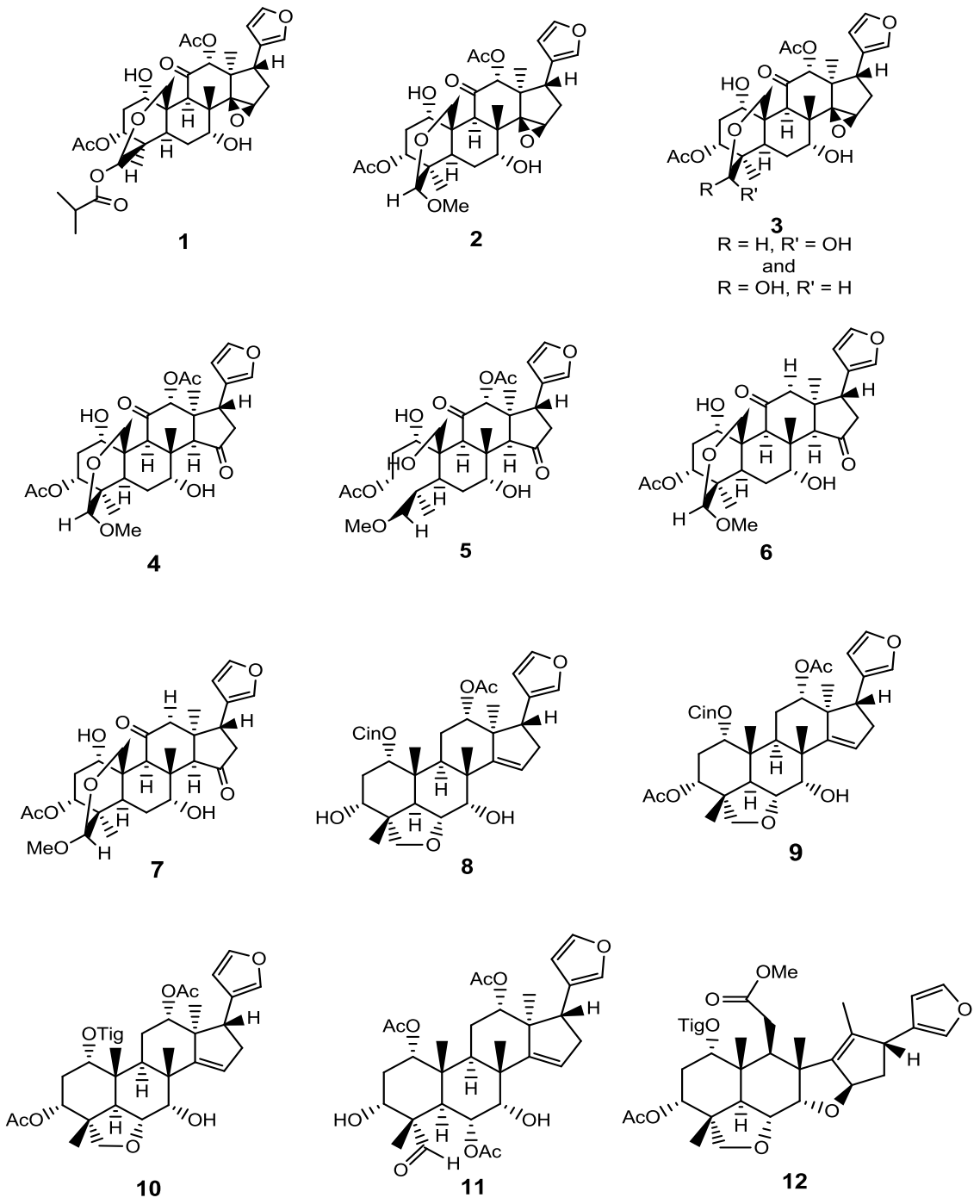

Figure 1. Structures of compounds 1-12

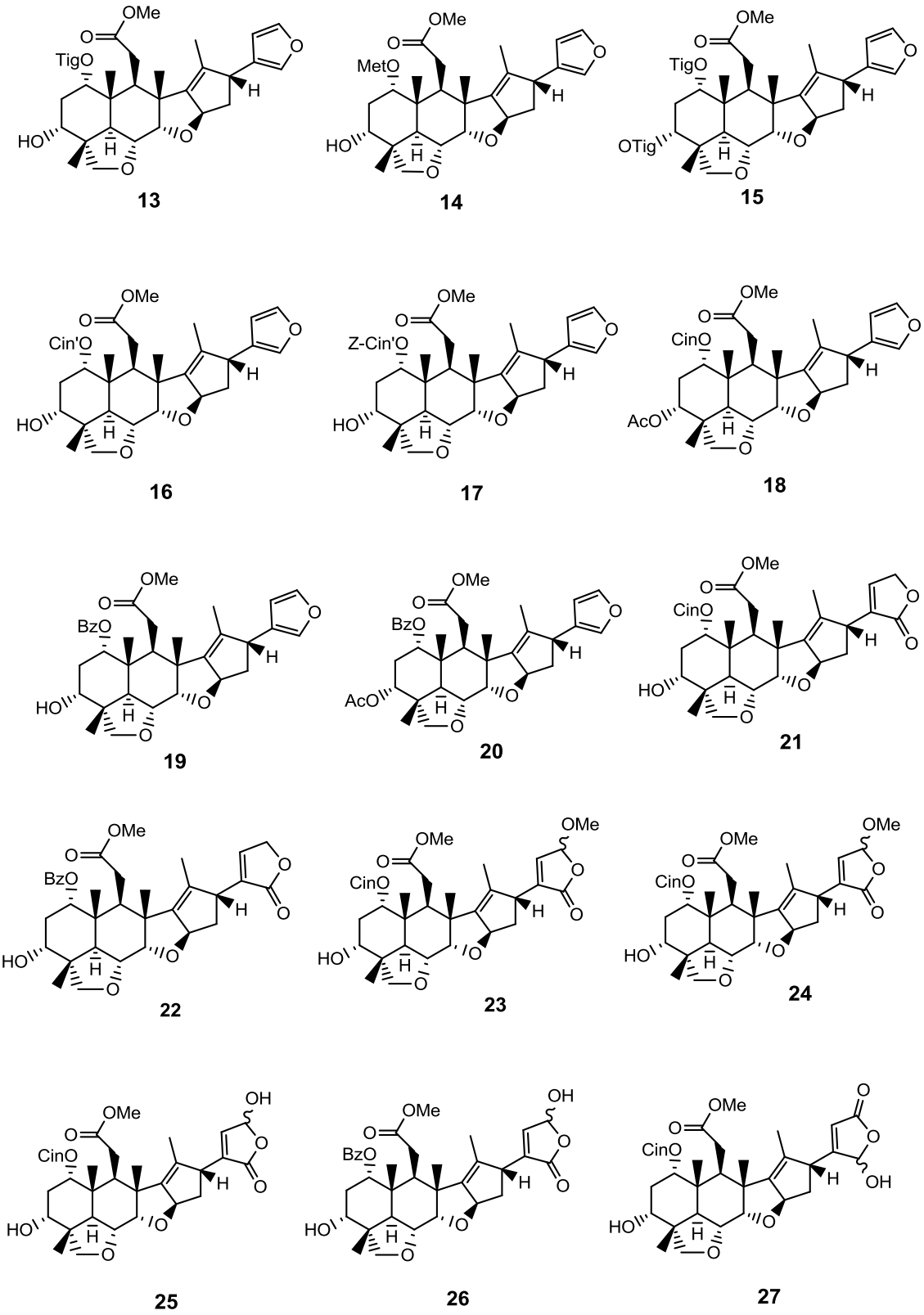

Figure 2. Structures of compounds 13-27

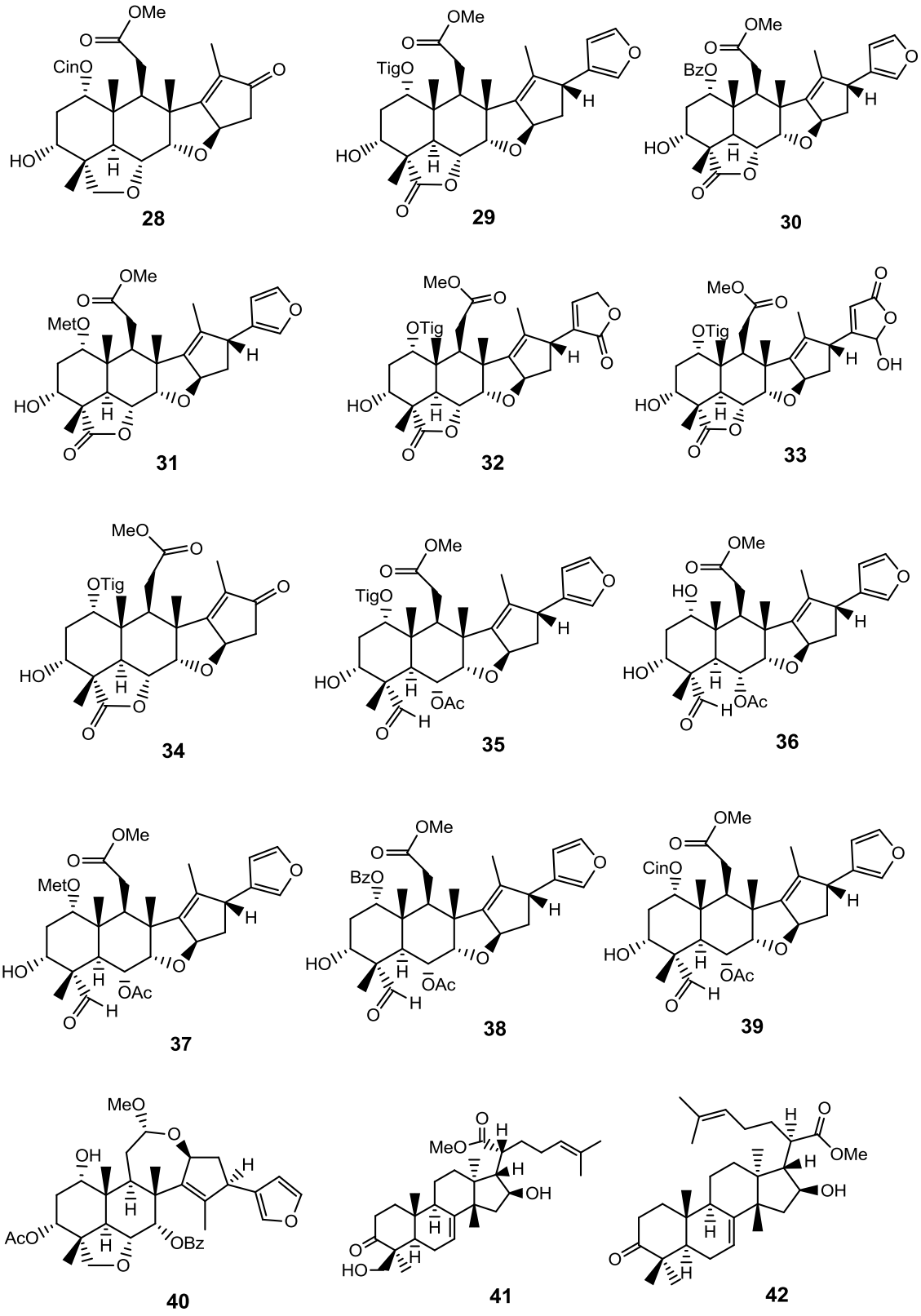

Figure 3. Structures of compounds 28-42

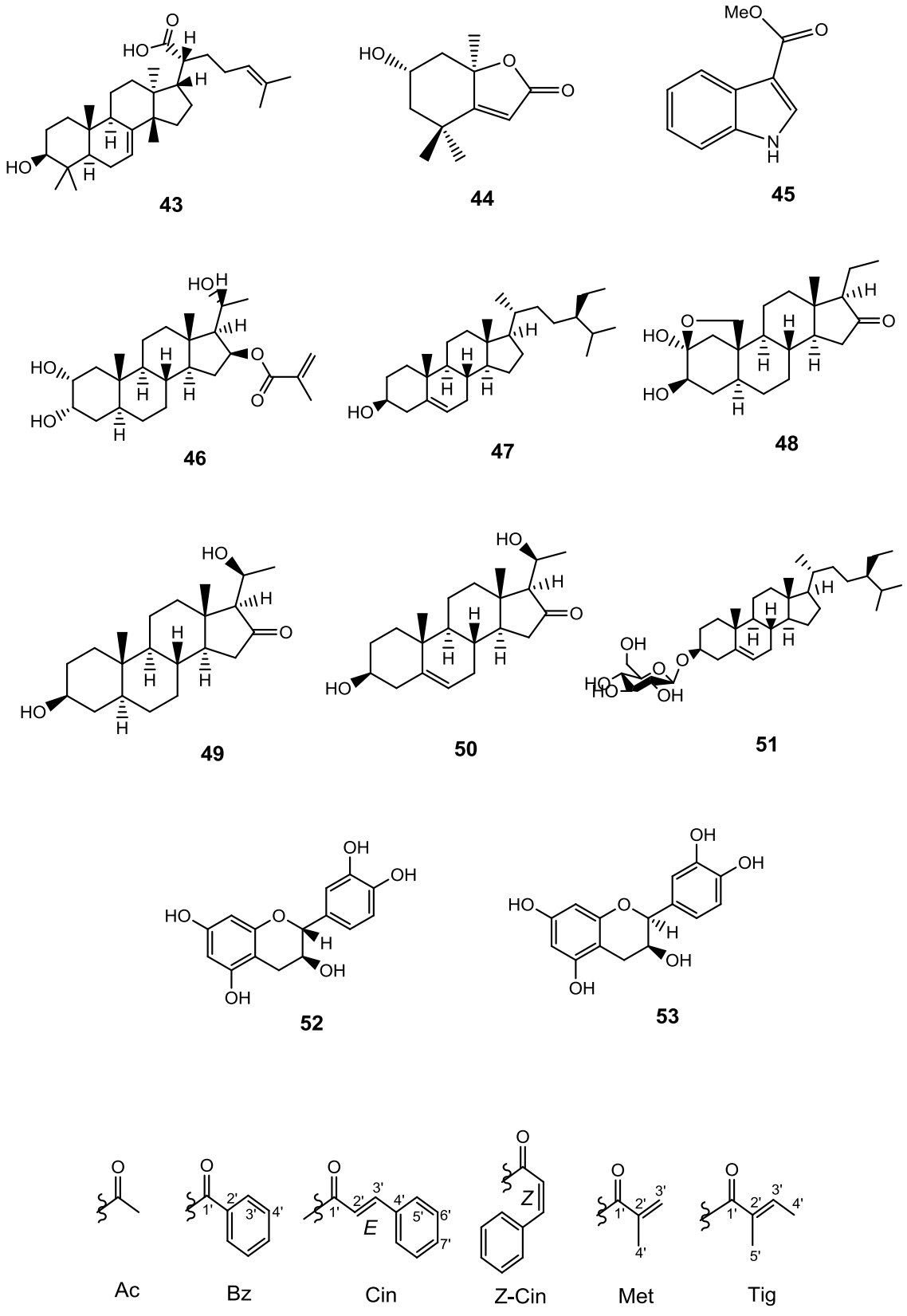

Figure 4. Structures of compounds 43-53

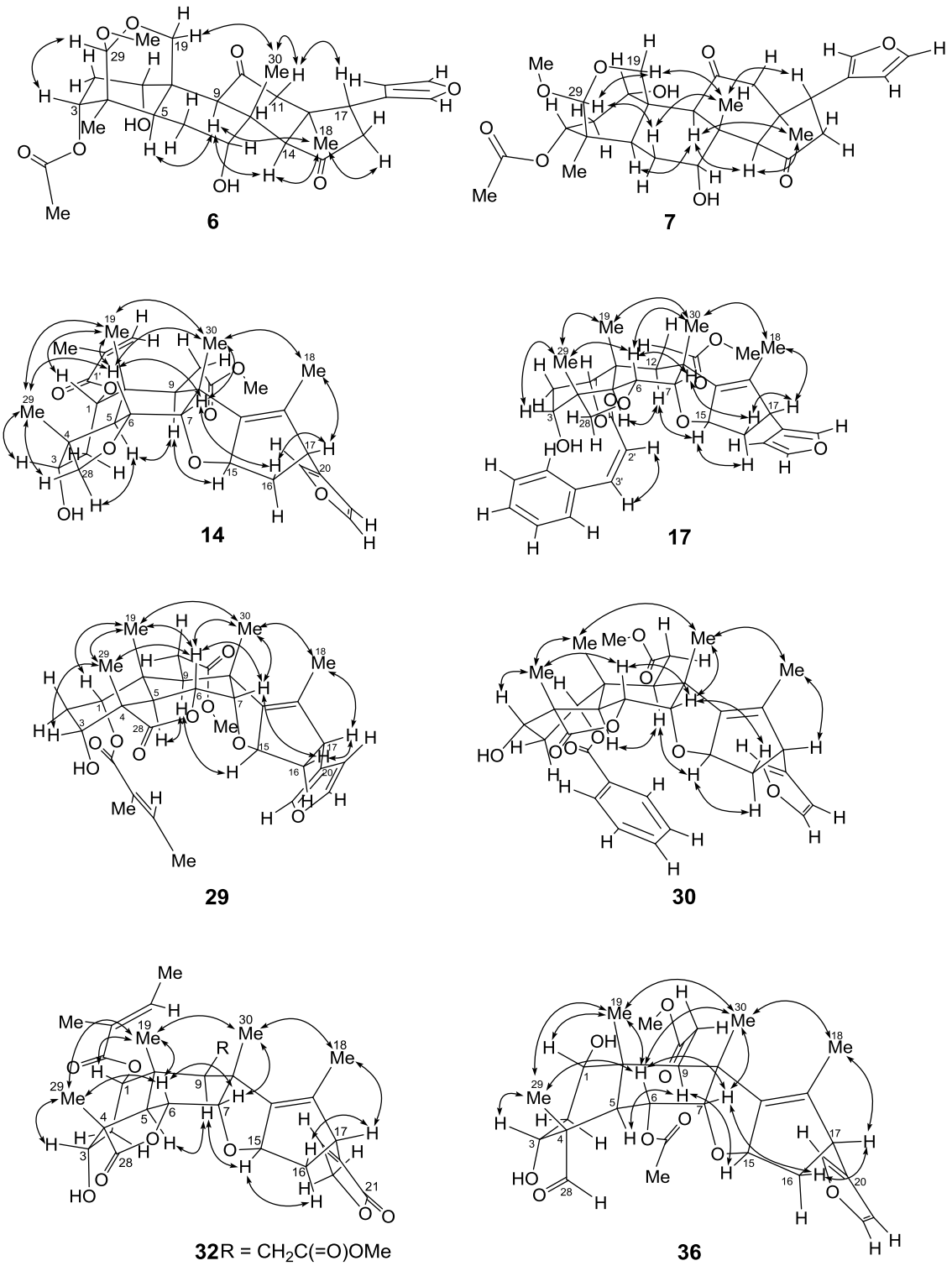

Figure 5. Major NOE correlation (↔) for compounds **6**, **7**, **14**, **17**, **29**, **30**, **32** and **36**. Drawings correspond to energy-minimized conformation of compounds. Calculation was performed using CAChe CONFLEX with the MM2 force field (CAChe version 5.5; Fujitsu Co., Tokyo, Japan).

Table 1.

^{1}H NMR Data of compounds **6, 7, 14, 17** and **19** from *Melia azedarach* (400 MHz, $CDCl_3$, δ in ppm, *J* in Hz).

Position	**6**	**7**	**14**	**17**	**19**
1	4.14 (br. d, 4.4)	4.08 (m)	5.04 (d, 8.0)	5.06 (t, 3.2)	5.22 (t, 3.2)
2	1.81 (α; br. d, 16.0)	1.81 (α; dt, 1.8, 16.0)	2.11–2.18 (α; m)	1.98 (α; dt, 3.0, 16.4)	2.13 (α; m)
	2.87 (β; dt, 4.6, 16.0)	2.85 (β; dt, 4.6, 16.0)	2.30–2.37 (β; m)	2.22 (β; m)	2.31 (β; m)
3	4.92 (br. d, 2.8)	5.35 (br. d, 2.8)	3.87–3.91 (m)	3.79 (m)	3.93 (t, 3.4)
5	2.28 (t, 7.6)	2.45 (br. dd, 2.8, 13.7)	2.72 (d, 12.8)	2.62 (d, 12.4)	2.90 (d, 12.4)
6	1.74 (α; dt, 3.5, 14.9)	1.78 (α; dt, 3.2, 13.8)	4.02 (dd, 3.2, 12.4)	4.00 (dd, 3.2, 12.4)	4.05 (dd, 3.2, 12.4)
	2.46 (β; dd, 2.5, 14.9)	2.02 (β; dd, 2.8, 14.2)			
7	3.89 (t, 2.7)	3.94 (m)	4.18 (d, 3.7)	4.19 (d, 3.2)	4.22 (d, 3.2)
9	3.42 (s)	3.45 (s)	2.64 (dd, 3.0, 9.4)	2.68 (dd, 2.5, 9.8)	2.72 (dd, 3.2, 10.1)
11			2.00–2.18 (m)	2.18 (m)	2.19 (m)
			2.24–2.30 (m)	2.34 (dd, 10.0, 15.6)	2.35 (m)
12	2.33 (α; d, 16.8)	2.33 (α; d, 17.0)			
	2.62 (β; d, 16.8)	2.65 (β; d, 17.0)			
14	3.12 (s)	3.15 (s)			
15			5.39 (br. t, 7.6)	5.50 (tq, 1.4, 6.8)	5.41 (tq, 1.4, 6.8)
16	2.55 (2H, d, 10.5)	2.58 (2H, d, 10.0)	2.23–2.27 (α; m)	2.28 (α; m)	2.25 (α; m)
			2.08–2.14 (β; m)	2.16 (β; m)	2.11 (β; m)
17	3.36 (dd, 4.8, 16.0)	3.36 (t, 10.6)	3.61 (br. d, 7.2)	3.63 (br. d, 8.7)	3.59 (br. d, 9.2)
18	0.96 (s)	0.99 (s)	1.65 (d, 1.4)	1.65 (d, 1.8)	1.64 (d, 1.4)
19	4.04 (d, 12.3)	4.09 (d, 12.8)	0.96 (s)	0.93 (s)	1.00 (s)
	4.34 (d, 12.3)	4.52 (d, 13.2)			
21	7.29 (br. s)	7.32 (br. s)	7.24 (br. s)	7.26 (d, 2.4)	7.08 (d, 0.9)
22	6.28 (br. s)	6.31 (dd, 1.2, 1.2)	6.26 (dd, 1.0, 2.0)	6.32 (dd, 0.8, 2.0)	6.07 (dd, 0.9, 0.9)
23	7.39 (t, 1.2)	7.42 (t, 1.2)	7.31 (t, 1.8)	7.31 (t, 1.6)	7.17 (t, 1.8)
28	0.85 (s)	0.87 (s)	3.64 (d, 7.3)	4.05 (α; d, 7.8)	4.20 (α; br. d, 7.8)
			4.13 (d, 7.3)	3.60 (β; d, 7.8)	3.67 (β; d, 7.3)
29	4.16 (s)	4.42 (s)	1.16 (s)	1.19 (s)	1.19 (s)
30	1.04 (s)	1.05 (s)	1.31 (s)	1.31 (s)	1.32 (s)
2'				6.10 (d, 12.4)	
3'			5.65 (t, 3.0)	7.05 (d, 12.4)	8.08 (dt, 1.4, 7.3)
			6.16 (t, 1.4)		
4'			2.05 (s)		7.50 (dt, 1.4, 7.8)
5'				7.62 (dt, 2.0, 7.2)	7.61 (dt, 1.8, 7.8)
6'				7.40 (tt, 1.6, 7.8)	
7'				7.36 (tt, 2.0, 7.8)	
AcO-3	2.09 (s)	2.09 (s)			
AcO-12				3.21 (s)	3.21 (s)
MeO-29	3.31 (s)	3.40 (s)			

Table 2.

^{1}H NMR Data of compounds **21–25** from *Melia azedarach* (400 MHz, $CDCl_3$, δ in ppm, *J* in Hz).

Position	**21**	**22**	**23**	**24**	**25**
1	5.06 (d, 2.8)	5.18 (d, 2.8)	5.06 (d, 3.0)	5.06 (d, 3.0)	5.14 (d, 2.8)
2	2.24 (α; m)	2.21 (α; m)	2.12 (α; m)	2.24 (α; m)	2.15 (α; m)
	2.34 (β; m)	2.40 (β; m)	2.25 (β; m)	2.36 (β; m)	2.38 (β; m)
3	3.91 (m)	3.91 (t, 3.2)	3.91 (t, 2.8)	3.90 (t, 2.7)	3.93 (t, 2.8)
5	2.76 (d, 12.4)	2.88 (d, 12.3)	2.76 (d, 12.4)	2.74 (d, 12.4)	2.75 (d, 12.4)
6	4.03 (dd, 3.7, 12.4)	4.03 (dd, 3.2, 12.4)	4.02 (dd, 3.2, 12.4)	4.03 (dd, 3.2, 12.4)	3.99 (dd, 3.2, 12.4)
7	4.25 (d, 3.7)	4.22 (d, 3.2)	4.23 (d, 3.2)	4.23 (d, 3.2)	4.24 (d, 3.2)
9	2.63 (dd, 3.7, 8.7)	2.67 (dd, 4.6, 7.8)	2.64 (dd, 3.6, 8.7)	2.64 (dd, 3.7, 8.4)	2.40 (m)
11	2.11 (m)	2.09 (m)	2.31 (m)	2.11 (m)	2.09 (m)
	2.34 (m)	2.37 (m)	2.39 (m)	2.24 (m)	2.33 (m)
15	5.36 (tq, 1.6, 6.4)	5.28 (tq, 2.2, 6.4)	5.31 (br. t, 7.2)	5.34 (br. t, 6.4)	5.31 (br. t, 7.6)
16	2.18 (α; m)	2.25 (α; m)	2.10 (2H, m)	2.25 (α; m)	2.19 (α; m)
	2.08 (β; m)	2.08 (β; m)		2.10 (β; m)	2.08 (β; m)
17	3.54 (br. d, 8.0)	3.50 (br. d, 8.7)	3.54 (br. d, 8.3)	3.53 (br. d, 8.8)	3.39 (m)
18	1.68 (d, 1.8)	1.68 (d, 1.4)	1.67 (d, 1.4)	1.70 (d, 1.3)	1.75 (d, 1.9)
19	1.00 (s)	1.03 (s)	0.99 (s)	0.99 (s)	0.93 (s)
22	7.06 (dd, 1.2, 2.8)	6.93 (dd, 1.4, 3.0)	6.83 (s)	6.75 (t, 1.4)	6.11 (t, 1.6)
23	4.27 (dt, 1.6, 18.0)	4.48 (dt, 1.4, 18.2)	5.24 (s)	5.57 (t, 1.2)	5.46 (br. d, 9.6)
	4.50 (dt, 1.6, 18.0)	4.54 (dt, 1.4, 18.2)			
28	4.16 (α; br. d, 7.8)	4.17 (α; d, 7.3)	4.15 (α; d, 7.8)	4.15 (α; d, 7.8)	4.20 (α; d, 7.8)
	3.66 (β; d, 7.8)	3.65 (β; d, 7.3)	3.66 (β; d, 7.8)	3.66 (β; d, 7.8)	3.66 (β; d, 7.8)
29	1.18 (s)	1.19 (s)	1.17 (s)	1.17 (s)	1.15 (s)
30	1.32 (s)	1.32 (s)	1.32 (s)	1.32 (s)	1.28 (s)
2'	6.46 (d, 16.0)		6.46 (d, 16.2)	6.46 (d, 16.0)	6.46 (d, 16.4)
3'	7.66 (d, 16.0)	8.04 (dt, 1.4, 6.8)	7.67 (d, 16.2)	7.68 (d, 16.0)	7.67 (d, 16.4)
4'		7.47 (t, 7.3)			
5'	7.55 (m)	7.59 (tt, 1.4, 7.3)	7.56 (m)	7.56 (m)	7.63 (m)
6'	7.42 (m)		7.44 (m)	7.41 (m)	7.41 (m)
7'	7.42 (m)		7.44 (m)	7.41 (m)	7.41 (m)
MeO-12	3.38 (s)	3.17 (s)	3.44 (s)	3.38 (s)	3.39 (s)
MeO-23			3.40 (s)	3.21 (s)	

Table 3.

^{1}H NMR Data of compounds **26**–**30** from *Melia azedarach* (400 MHz, $CDCl_3$, δ in ppm, *J* in Hz).

Position	**26**	**27**	**28**	**29**	**30**
1	5.28 (t, 3.2)	4.99 (m)	5.19 (t, 3.0)	4.88 (d, 3.0)	5.08 (t, 2.8)
2	2.11 (α; m)	2.40 (α; m)	2.13 (α; dt, 2.7, 16.5)	2.14–2.22 (α; m)	2.18 (α; m)
	2.38 (β; m)	2.28 (β; m)	2.36 (β; dt, 2.7, 15.1)	2.25–2.32 (β; m)	2.25 (β; m)
3	3.97 (t, 2.8)	3.91 (t, 3.2)	3.95 (dt, 2.7, 8.2)	4.13 (dd, 4.3, 6.2)	4.17 (t, 2.8)
5	2.87 (d, 12.8)	2.73 (d, 12.3)	2.79 (d, 12.8)	3.39 (d, 12.4)	3.56 (d, 12.4)
6	4.03 (dd, 3.2, 12.4)	4.03 (dd, 3.2, 12.4)	4.09 (dd, 3.4, 12.7)	4.52 (dd, 3.4, 12.6)	4.56 (dd, 3.2, 12.3)
7	4.27 (d, 3.2)	4.26 (d, 3.2)	4.17 (d, 3.7)	4.25 (d, 3.7)	4.27 (d, 3.2)
9	2.47 (br. d, 10.6)	2.66 (m)	2.83 (t, 3.2)	2.83 (dd, 3.9, 8.9)	2.87 (dd, 3.9, 8.9)
11	2.24 (m)	2.12 (m)	2.36 (dd, 2.3, 15.1)	2.13–2.22 (m)	2.21 (m)
	2.45 (m)	2.32 (m)	2.44 (dd, 10.5, 15.6)	2.26–2.35 (m)	2.32 (m)
15	5.31 (br. t, 6.4)	5.56 (t, 6.9)	5.11 (tt-like, 1.8, 6.2)	5.45 (t, 7.6)	5.42 (br. t, 6.8)
16	2.29 (α; m)	2.15 (α; m)	2.48 (α; dd, 3.6, 17.4)	2.20–2.26 (α; m)	2.28 (α; m)
	2.09 (β; m)	2.39 (β; m)	2.85 (β; dd, 3.2, 17.0)	2.06–2.16 (β; m)	2.13 (β; m)
17	3.43 (br. d, 6.4)	3.77 (br. d, 9.2)		3.65 (d, 8.7)	3.60 (br. d, 8.4)
18	1.75 (d, 1.8)	1.68 (s)	1.75 (d, 1.8)	1.68 (s)	1.66 (d, 1.4)
19	0.98 (s)	1.02 (s)	1.04 (s)	1.03 (s)	1.07 (s)
21		5.81 (s)		7.25 (s)	7.06 (s)
22	6.19 (br. s)	5.90 (s)		6.28 (br. s)	6.03 (br. s)
23	5.66 (d, 10.3)			7.33 (br. s)	7.16 (t, 1.6)
28	4.23 (α; d, 7.3)	4.14 (α; br. d, 7.8)	3.70 (β; d, 7.3)		
	3.68 (β; d, 7.3)	3.66 (β; d, 7.8)	4.19 (α; br. d, 7.8)		
29	1.19 (s)	1.17 (s)	1.19 (s)	1.25 (s)	1.29 (s)
30	1.30 (s)	1.30 (s)	1.41 (s)	1.36 (s)	1.37 (s)
2'		6.44 (d, 16.0)	6.47 (d, 16.0)		
3'	8.02 (dt, 1.4, 7.3)	7.68 (d, 16.0)	7.70 (d, 16.0)	6.95 (dd, 6.0, 13.6)	8.12 (d, 7.8)
4'	7.54 (t, 7.3)			1.82 (d, 6.9)	7.46 (t, 7.6)
5'	7.65 (tt, 1.4, 7.3)	7.56 (m)	7.54 (dd, 3.6, 7.8)	1.90 (s)	7.57 (d, 7.8)
6'		7.42 (m)	7.41 (m)		7.46 (t, 7.6)
7'		7.42 (m)	7.41 (m)		8.12 (d, 7.8)
MeO-12	3.31 (s)	3.52 (br. s)	3.25 (s)	3.22 (s)	2.76 (s)

Table 4.

^{1}H NMR Data of compounds **31–34**, and **36** from *Melia azedarach* (400 MHz, $CDCl_3$, δ in ppm, *J* in Hz).

Position	**31**	**32**	**33**	**34**	**36**
1	4.87 (t, 3.0)	4.82 (t, 2.8)	4.76 (t, 2.8)	5.00 (t, 3.0)	3.66–3.68 (m)
2	2.14 (α; m)	2.06–2.12 (α; m)	2.04–2.12 (α; m)	2.10 (α; t, 2.5)	1.97–2.10 (2H, m)
	2.23 (β; m)	2.10–2.20 (β; m)	2.13–2.21 (β; m)	2.17 (β; t, 3.2)	
3	4.13 (t, 2.8)	4.12 (br.s)	4.12 (t, 2.8)	4.18 (t, 2.8)	3.72 (br. t, 8.0)
5	3.39 (d, 12.4)	3.36 (d, 12.4)	3.32 (d, 12.4)	3.42 (d, 12.4)	3.60 (d, 12.4)
6	4.52 (dd, 3.5, 12.5)	4.51 (dd, 3.6, 12.6)	4.50 (dd, 3.4, 12.6)	4.57 (dd, 3.4, 12.6)	5.22 (dd, 3.2, 12.3)
7	4.24 (d, 3.6)	4.28 (d, 3.6)	4.33 (d, 3.4)	4.21 (d, 3.7)	4.11 (d, 2.7)
9	2.84 (dd, 4.0, 9.0)	2.78 (dd, 5.0, 7.3)	2.77 (t, 5.7)	2.94 (dd, 3.2, 10.5)	2.68 (br. t, 4.6)
11	2.18 (m)	2.24 (dd, 6.4, 12.4)	2.34 (dd, 6.7, 12.4)	2.32 (dd, 3.2, 15.6)	2.35 (dd, 4.1, 17.4)
	2.34 (m)	2.34 (dd, 7.3, 15.1)	2.46 (dd, 6.4, 15.6)	2.42 (dd, 10.5, 15.6)	2.46 (dd, 5.5, 17.4)
15	5.46 (br. t, 7.2)	5.35 (dt, 1.8, 6.8)	5.60 (br. t, 6.9)	5.11 (dt-like, 2.3, 4.1)	5.50 (br. t, 7.6)
16	2.27 (α; m)	2.19–2.26 (α; m)	2.35–2.42 (2H, m)	2.47 (α; dd, 3.4, 16.9)	2.25 (α; dd, 6.4, 11.4)
	2.10 (β; m)	2.06–2.11 (β; m)		2.87 (β, dd, 6.4, 16.9)	2.00–2.08 (β; m)
17	3.65 (br. d, 8.7)	3.60 (br. d, 8.7)	3.85 (br. d, 9.2)		3.64 (br. d, 7.6)
18	1.68 (d, 1.6)	1.72 (d, 1.4)	1.73 (d, 1.4)	1.78 (d, 1.8)	1.71 (d, 1.3)
19	1.03 (s)	1.05 (s)	1.08 (s)	1.07 (s)	0.98 (s)
21	7.23 (s)		5.80 (br. s)		7.12 (s)
22	6.27 (br. s)	7.28 (d, 0.9)	5.97 (br. s)		6.11 (s)
23	7.32 (t, 1.6)	4.77 (2H, br. s)			7.31 (t, 1.2)
28					9.68 (s)
29	1.25 (s)	1.25 (s)	1.26 (s)	1.28 (s)	0.93 (s)
30	1.36 (s)	1.36 (s)	1.34 (s)	1.46 (s)	1.36 (s)
3'	5.60 (t, 1.6)	6.92 (dq, 1.4, 6.9)	6.91 (dq, 1.4, 6.9)	6.97 (dq, 1.4, 7.3)	
	6.22 (br. s)				
4'	2.03 (s)	1.80 (dd, 1.4, 7.1)	1.82 (dd, 1.3, 7.1)	1.82 (dd, 1.2, 7.1)	
5'		1.86 (t, 1.4)	1.84 (br. s)	1.87 (t, 1.4)	
AcO-6					1.95 (s)
MeO-12	3.24 (s)	3.43 (s)	3.58 (s)	3.35 (s)	3.58 (s)

Table 5.

^{13}C NMR data of ten compounds (**6**, **7**, **14**, **17**, **19**, **21**-**25**) from *Melia azedarach* (100 MHz, $CDCl_3$, δ in ppm).

Position	**6**	**7**	**14**	**17**	**19**	**21**	**22**	**23**	**24**	**25**
1	70.7	70.8	73.1	72.6	73.3	73.2	73.4	73.1	73.0	72.6
2	36.2	35.2	30.0	30.5	30.9	30.4	30.2	30.5	30.6	30.2
3	76.7	74.4	70.8	70.7	70.7	70.8	70.7	70.8	70.7	70.7
4	40.4	39.7	44.2	44.1	44.2	44.2	44.2	44.1	44.1	44.2
5	26.0	28.1	38.8	39.0	38.9	39.3	38.8	38.7	38.8	38.5
6	25.0	23.1	72.5	72.4	73.5	72.3	72.3	72.2	72.2	72.2
7	69.6	69.4	85.7	85.7	85.9	86.0	86.0	86.0	85.8	86.1
8	45.0	44.8	49.0	49.0	48.9	49.0	49.0	49.0	49.1	48.1
9	52.1	51.8	39.5	39.4	39.7	38.7	39.5	39.0	39.1	39.1
10	43.4	41.6	40.8	40.6	41.2	41.0	41.1	40.9	40.8	41.3
11	211.0	210.6	30.5	30.1	30.8	30.5	30.7	30.4	30.5	30.8
12	50.8	50.5	172.7	172.8	172.5	173.1	172.9	173.0	172.9	174.3
13	43.3	41.6	135.0	134.8	135.4	134.1	132.8	132.0	132.1	132.8
14	62.3	62.8	146.4	146.4	146.2	148.7	148.6	149.3	149.1	147.3
15	219.6	219.7	87.9	87.8	88.0	87.4	87.4	87.2	87.2	87.3
16	42.1	43.3	41.2	41.0	41.1	40.0	39.9	39.7	39.8	40.0
17	41.8	41.0	49.3	49.3	49.3	48.6	48.7	48.3	48.6	48.7
18	28.0	28.0	13.1	13.0	13.3	13.1	13.2	13.0	13.2	13.2
19	58.3	63.4	15.3	15.5	15.2	15.2	15.1	15.2	15.2	15.1
20	122.0	121.8	127.1	127.1	127.1	135.1	135.0	139.3	139.4	137.0
21	140.2	140.1	138.8	138.8	138.4	174.3	174.2	171.4	171.3	171.3
22	110.3	110.2	110.7	110.8	110.6	145.0	144.8	142.6	142.3	141.7
23	143.4	143.3	142.9	142.8	142.8	70.0	70.0	102.6	102.1	96.7
28	18.3	19.5	77.8	77.8	77.9	77.9	77.9	77.9	77.8	77.9
29	102.7	102.8	19.8	19.6	20.0	19.9	20.0	19.9	19.1	19.8
30	20.4	20.1	16.9	17.0	16.9	16.7	16.8	16.8	16.8	16.1
1'			165.0	164.8	165.1	165.2	164.9	165.3	165.4	164.7
2'			136.2	119.5	130.2	117.1	130.2	117.7	117.6	117.6
3'			126.2	143.7	129.6	145.3	129.6	145.3	145.5	145.3
4'			18.4	134.6	128.8	132.7	128.5	134.2	134.0	133.8
5'				129.7	133.3	128.1	133.1	128.2	128.1	128.6
6'				128.2		129.0		128.9	129.0	128.9
7'				129.3		130.7		130.6	130.6	130.8
AcO-3	21.4 169.8	21.4 169.3								
MeO-12			51.5	51.5	51.5	51.5	51.3	51.9	51.6	52.6
AcO-12										
MeO-23								57.1	55.6	
MeO-29	55.6	56.9								

Table 6.

^{13}C NMR data of ten compounds (**26**-**34**, **36**) from *Melia azedarach* (100 MHz, CDCl3, δ in ppm).

Position	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**36**
1	72.7	73.3	72.5	71.3	71.7	71.5	71.4	71.6	70.7	72.5
2	31.1	30.3	30.8	29.4	29.7	29.3	29.2	28.9	29.8	29.0
3	70.5	70.8	70.6	68.9	68.8	68.8	68.8	68.8	68.7	76.2
4	44.2	44.2	44.2	48.9	47.7	47.6	47.6	47.6	47.6	49.4
5	38.8	38.9	39.0	37.4	37.3	37.4	38.8	37.5	37.6	34.0
6	72.4	72.3	72.1	74.3	74.3	74.3	74.1	74.0	73.7	69.0
7	86.1	86.0	85.0	83.1	83.2	83.2	83.2	83.4	82.3	86.3
8	48.2	48.1	50.0	47.7	48.7	49.0	49.1	49.5	50.0	47.4
9	39.2	39.2	40.6	38.7	38.8	38.6	38.6	38.8	39.6	40.1
10	41.3	40.9	41.1	40.3	40.5	40.2	40.3	40.3	40.4	42.9
11	30.1	30.8	30.5	30.8	31.2	30.7	30.8	31.4	30.7	30.9
12	174.3	174.4	172.2	172.4	172.0	172.4	172.9	174.7	171.9	177.2
13	132.8	130.6	134.1	135.9	136.0	136.0	133.6	131.4	134.5	135.4
14	147.3	150.5	176.5	145.5	145.0	145.4	147.9	149.7	175.2	146.4
15	87.3	88.2	78.8	88.4	88.4	88.4	87.9	88.9	79.1	87.5
16	40.1	39.0	45.8	41.0	40.9	40.9	39.7	39.3	45.7	41.5
17	48.7	52.8	206.1	49.4	49.3	49.4	48.8	53.0	205.5	49.6
18	13.2	13.0	8.2	13.0	13.2	13.0	13.2	13.0	8.2	12.9
19	15.2	15.2	15.4	15.5	15.4	15.6	15.5	15.2	15.7	16.3
20	137.1	169.0		126.8	126.6	126.7	135.2	168.8		126.8
21	171.4	97.7		138.8	138.8	138.8	174.1	97.5		138.6
22	141.5	119.8		110.5	110.3	110.5	144.9	120.4		110.9
23	96.6	170.3		143.0	142.7	143.0	70.2	169.8		143.3
28	77.9	78.0	78.0	177.5	177.6	177.5	177.4	177.4	177.2	208.0
29	19.9	20.0	19.6	16.6	15.6	15.0	15.5	15.7	15.4	13.8
30	16.2	16.9	16.3	15.6	16.5	16.6	16.6	16.6	16.1	17.4
1'	164.7	165.5	165.5	166.7	165.0	166.2	166.7	166.5	166.6	
2'	129.8	117.4	117.1	128.8	130.3	136.3	128.7	128.5	128.5	
3'	129.4	146.1	140.3	137.9	129.0	126.0	137.8	138.7	138.2	
4'	128.8	133.9	133.9	12.0	128.3	18.2	14.6	14.6	14.6	
5'	133.4	128.4	128.4	14.5	133.0		12.0	11.9	12.1	
6'		129.1	129.1							
7'		130.9	131.0							
AcO-3										
ACO-6										21.0, 170.4
MeO-12	52.5	52.5	51.6	51.5	51.5	51.5	51.6	52.8	51.6	51.7
AcO-12										
MeO-23										
MeO-29										

Chapter 4

Biological activities of Constituents from *Melia azedarach*

4.1 Inhibitory Effects on TPA-Induced EBV-EA Activation

The inhibitory effects on the induction of EBV-EA induced by TPA were further examined as a preliminary evaluation of the potential anti-tumor-promoting effects [19] for thirty-nine compounds (**2**–**9**, **16**, **17**, **19**–**35**, **40**, **41**, **46-53**). The results are compiled in Table 7, together with comparable data for β-carotene, a vitamin A precursor studied widely in cancer chemoprevention animal models, along with that of compound (**52**) [66] which was examined in our recent study. All of the compounds tested exhibited moderate inhibitory effects with IC_{50} values (concentration of 50% inhibition with respect to positive control) in the range of 299–530 mol ratio/32 pmol TPA with preservation of high viability (60%) of Raji cells. Among the compounds tested, nine compounds (**2**, **7**, **33**, **34**, **37**, **46**, **51-53**) including five limonoids (**2**, **7**, **33**, **34**, **37**) which three limonoids (**7**, **33**, **34**) were new compounds, exhibited more potent than or almost equivalent inhibitory effects with the reference compound, β-carotene (IC_{50} 397 mol ratio/32 pmol TPA). Since the inhibitory effects against EBV-EA activation have been demonstrated to closely parallel to those against tumor promotion *in vivo* [19], nine compounds (**2**, **7**, **33**, **34**, **37**, **46**, **51-53**) are potential inhibitors of tumor promotion.

Table 7

Inhibitory Effects on the Induction of Epstein–Barr Virus Early Antigen (EBV-EA) of Thirty-Eight Compounds from *Melia azedarach* Extracts.

NO.	Compound	Percentage EBV-EA induction[a])				IC$_{50}$[c])
		Drug conentration[b])				
		1000	500	100	10	
2	Meliarachin C	2 ± 0.3 (60)	41 ± 1.7	70 ± 2.1	95 ± 0.5	347
3	Toosendanin	7.0 ± 0.4 (60)	45 ± 1.7	74 ± 2.2	97 ± 0.6	432
4	Meliarachin K	9 ± 0.5 (60)	47 ± 1.6	75 ± 2.2	98 ± 0.4	450
5	Meliarachin G	8 ± 0.4 (60)	46.0 ± 1.6	73 ± 2.1	100 ± 0.4	440
6	12-Dehydroneoazedarachin D	9 ± 0.6 (60)	48 ± 1.7	75 ± 2.2	100 ± 0.4	452
7	12-Dehydro-29-exo-neoazedarachin D	4 ± 0.3 (60)	43 ± 1.5	72 ± 2.1	98 ± 0.5	401
8	Trichilinin D	11 ± 1.2 (60)	50 ± 1.8	77 ± 2.3	100 ± 0.3	496
9	1-*O*-Cinnamoyltrichilinin	14 ± 1.1 (60)	52 ± 1.8	78.0 ± 2.3	100 ± 0.4	529
16	Ohchinin	11 ± 0.9 (60)	50 ± 1.7	75 ± 2.2	100 ± 0.4	452
17	1-*O*-Decinnamoyl-1-*O*-cis-cinnamoylohchinin	11 ± 0.8 (60)	49 ± 1.7	75 ± 2.3	100 ± 0.4	496
19	1-*O*-Decinnamoyl-1-*O*-benzoylohchinin	13 ± 0.9 (60)	51 ± 1.8	77 ± 2.4	100 ± 0.4	482
20	1-*O*-Decinnamoyl-1-*O*-benzoylohchinin acetate	8 ± 0.6 (60)	46.0 ± 1.6	73 ± 2.1	100 ± 0.5	449
21	Ohchininolide	14 ± 1.1 (60)	51 ± 1.8	78 ± 2.4	100 ± 0.4	521
22	1-*O*-Decinnamoyl-1-*O*-benzoylohchininolide	11 ± 1.0 (60)	49 ± 1.7	76 ± 2.3	100 ± 0.4	495
23	23-Methoxyohchininolide A	15 ± 1.2 (60)	53 ± 1.8	80 ± 2.5	100 ± 0.3	530
24	23-Methoxyohchininolide B	14.0 ± 1.1 (60)	52 ± 1.8	79 ± 2.5	100 ± 0.3	528
25	23-Hydroxyohchininolide	12 ± 0.5 (60)	50 ± 1.4	77 ± 2.5	100 ± 0.4	497
26	1-*O*-Decinnamoyl-1-*O*-benzoyl-23-hydroxyohchininol	11 ± 1.2 (60)	49 ± 1.8	76 ± 2.3	100 ± 0.4	493
27	21-Hydroxyisoohchininolide	9.0 ± 1.0 (60)	47 ± 1.6	74 ± 2.4	100 ± 0.5	453
28	17-Defurano-17-oxoohchinin	12 ± 1.1 (60)	50 ± 1.9	77 ± 2.6	100 ± 0.3	497
29	3-Deacetyl-28-oxosalannin	10 ± 0.5 (60)	50 ± 1.5	77 ± 2.3	100 ± 0.3	473
30	1-*O*-Decinnamoyl-1-*O*-benzoyl-28-oxoohchinin	10 ± 1.0 (60)	47 ± 1.7	74 ± 2.6	100 ± 0.4	488
31	3-Deacetyl-4'-demethyl-28-oxosalannin	2.5 ± 0.3 (60)	43.6 ± 1.6	72.0 ± 2.4	98.7 ± 0.6	425
32	3-Deacetyl-28-oxosalannolactone	3.2 ± 0.4 (60)	45.3 ± 1.5	73.7 ± 2.3	100 ± 0.3	431
33	3-Deacetyl-28-oxoisosalanninolide	4 ± 0.6 (60)	38 ± 1.4	69.4 ± 2.3	95.5 ± 0.4	299
34	3-Deacetyl-17-defurano-17,28-dioxosalannin	4 ± 0.5 (60)	39 ± 1.3	73.6 ± 2.3	98.7 ± 0.5	318
35	Ohchinolal	4.2 ± 0.4 (60)	45.2 ± 1.5	72.8 ± 2.1	98.9 ± 0.5	423
37	Mesendanin E	3 ± 0.4 (60)	44 ± 1.6	71 ± 2.2	95 ± 0.5	378
40	Nimbolinin D	3.6 ± 0.5 (60)	40.8 ± 1.5	70.6 ± 2.1	98.8 ± 0.6	429
41	Meliasenin E	0.0 ± 0.3 (70)	46 ± 1.6	76 ± 2.4	95 ± 0.6	417
44	Loliolide	14 ± 0.7 (60)	51 ± 1.5	81.7 ± 2.6	100 ± 0.3	488
46	2α,3α,20*R*-Trihydroxy-5α-pregnane 16β-methacrylate	4 ± 0.4 (70)	39 ± 1.4	71.1 ± 2.4	97.8 ± 0.5	310
47	Sitosterol	14 ± 0.7 (70)	49 ± 1.5	80.6 ± 2.5	100 ± 0.4	479
48	2,19-Oxymeliavosin	7 ± 0.5 (70)	44 ± 1.4	70.6 ± 2.3	100 ± 0.5	451
49	Toosendansterol A	8 ± 0.4 (70)	46 ± 1.4	73.8 ± 2.3	100 ± 0.5	469
50	5,6-Dehydrotoosendansterol A	8 ± 0.6 (70)	46 ± 1.5	72.1 ± 2.4	100 ± 0.4	461
51	Sitosterol 3-*O*-β-D-glucopyranoside	3 ± 0.3 (70)	28 ± 1.0	75.3 ± 2.0	100 ± 0.3	361
52	(+)-Catechin	2 ± 0.3 (60)	40 ± 1.3	72 ± 2.3	98.0 ± 0.5	352
53	(-)-Epicatechin	4 ± 0.3 (60)	42 ± 1.3	73 ± 2.3	99 ± 0.5	381
β-Carotene[d])		8.6 ± 0.5 (70)	34.2 ± 1.0	82.1 ± 2.0	100 ± 0.3	397

[a])Values represent the relative percentage to the positive control, with TPA (32 pmol, 20 ng) representing 100% induction. Data are expressed as mean ± S.D. (n = 3). [b]) Concentration in terms of molar ratio/32 pmol TPA. [c]) IC$_{50}$ represents the molar ratio of compound, relative to TPA, required to inhibit 50% of the positive control activated with 32 pmol TPA. [d]) Reference compound.

4.2 Cytotoxic Activity against Four Human Cancer Cell Lines

4.2.1 Cytotoxic Activity of the Extracts of *Melia azedarach* Fruits

The hexane extract and the defatted more-polar fraction, the MeOH extract and the three fractions from the MeOH extract were evaluated for their cytotoxic activities

against four human cancer cell lines: HL60 (leukemia), A549 (lung), AZ521 (duodenum), and SK-BR-3 (breast), by means of a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-2*H*-tetrazolium bromide (MTT) assay, and the results are summarized in Table 8. The hexane extract was inactive against all cell lines tested, the defatted hexane fraction exhibited cytotoxic activities against all cell lines with IC_{50} values in the range of 2.9–21.9 μg/ml. While the EtOAc-soluble fraction exhibited potent activity against HL60, A549, and AZ521 cells, and the *n*-BuOH-soluble fraction against HL60 and AZ521 cells. The EtOAc-soluble fraction and the defatted fraction were further investigated for their constituents in this study.

4.2.2 Cytotoxic Activity of the Extracts of *Melia azedarach* Leaves and Bark

All extracts and the fractions of *M. azedarach* leaves and bark were evaluated for their cytotoxic activities against four human cancer cell lines, HL60 (leukemia), A549 (lung), AZ521 (duodenum), and SK-BR-3 (breast), by means of MTT assay, and the results are summarized in Table 8. The leaves and bark MeOH extract exhibited potent (IC_{50} 7.5–13.6 μg/ml) and moderate (IC_{50} 37.7–81.6 μg/ml) cytotoxic activities against HL60, AZ521, and SK-BR-3 cells, respectively. The EtOAc-soluble fraction of the leaves MeOH extract also exhibited potent cytotoxicities (IC_{50} 9.3–26.2 μg/ml) against the above three cell linens, while the EtOAc-soluble fraction of the bark MeOH extracts showed cytotoxicity only against HL60 cells (IC_{50} 23.1 μg/ml). The H_2O-soluble fractions of both leaves and bark MeOH extract exhibited no, or almost no, activity against the three cells. A549 cells were insensitive to all of the extract and fractions tested.

4.2.3 Cytotoxic Activity of the Compounds from *Melia azedarach*

Fifty-three compounds (**1**–**53**) isolated from *M. azedarach* fruits, leaves and bark,

which twenty limonoids (**6**, **7**, **14**, **17**, **19**, **21-34**, **36**) were new compounds, were evaluated against four human cancer cell lines by MTT assay, and the results are summarized in Table 9 and Table 10. Fifteen limonoids (**1-3**, **7**, **9**, **11**, **14**, **18**, **23**, **26**, **31**, **36**, **38-40**) and three compounds (**41-43**) exhibited IC_{50} values of 0.005–9.9 μM against one or more cancer cell lines. Compounds (**1-3**, **7**, **9**, **11**, **14**, **18**, **23**, **31**, **36**, **38**–**43**) against HL60 cell line, compounds (**1-3**, **31**, **36**) against AZ521 cell line, compound (**26**) against SK-BR-3 cell line, and compound (**42**) against A549 showed potent activities. In addition, the cytotoxicities of compounds (**6**, **41**) against AZ521 cells, compounds (**19**, **41**, **42**) against SK-BR-3, and compounds (**1**, **36**) against A549 were observed to be almost comparable with those of the reference cisplatin (AZ521: IC_{50} 9.5 μM; SK-BR-3: IC_{50} 18.8 μM; A549: IC_{50} 18.4 μM). The lung cancer cells (A549) were less sensitive to the compounds tested, and compounds (**2**, **18**–**20**, **26**, **38**, **41**, **46**) showed weak cytotoxicities (IC_{50} 42.3–94.3 μM), being less active than cisplatin (IC_{50} 18.4 μM).

Among the fifty three compounds (**1**–**53**) described in this paper, potent cytotoxicities of compounds (**1**) against P388 mouse lymphocytic leukemia cell line (ED_{50} 0.034 μg/ml) [67], compound (**3**) against HL60, PC3 (prostate), BEL7404 (liver), SH-SY5Y (CNS), U251 (CNS), and U937 (histocyte) cells [68], and compound (**42**) against P388 mouse lymphocytic leukemia cell line (ED_{50} 5.1 μg/ml) [69], and U20S human osteosarcoma (IC_{50} 14.6 μg/ml) and MCF-7 human breast cancer cell lines (IC_{50} 0.41 μg/ml) [70], and compound (**46**) against HGC27 duodenum cancer cell line (IC_{50} 11.3 μg/ml) [11] and no cytotoxicity of compounds (**38**, **39**) against HeLa S3 (epithelial) cells [43] have previously been reported.

Based on the results in Tables 8-10, it is highly possible that compounds (**1-3**, **7**, **9**, **11**, **14**, **18**, **23**, **26**, **31**, **36**, **38**–**43**) are at least in part, responsible for the cytotoxicities of the defatted fraction of the hexane extract of *M. azedarach* fruits, the EtOAc-soluble fraction of the MeOH extract of *M. azedarach* fruits and the EtOAc -soluble fraction of the MeOH extract of *M. azedarach* leaves against HL60, AZ521, and SK-BR-3 cell lines.

On the basis of the results in Table 9 and Figure 5, the following conclusions can be drawn about the structure-activity relationship of the compounds evaluated:

(i) Among the trichilin-type limonoids, those compounds (**1-3**) with a β-oriented epoxy-ring at C-14/C-15 are far more active than those of their 15-oxo analogues (**4**–**7**) against HL60 and AZ521 cells. Furthermore, deacetoxylation at C-12 increased the activity against HL60 and AZ521 cells among the 15-oxo trichilins (**6** *vs*. **4** and **7** *vs*. **5**). Potent cytotoxicity against HL60 cells of the compounds possessing a C-14/C-15 β–epoxy-ring was observed also for some azadiradione- and gedunin-type limonoids [26].

(ii) Acetylation of the hydroxy group at C-3 of vilasanin- (**9** *vs.* **8**) and salannin-type limonoids (**18** *vs*. **16**/**17**; **20** *vs*. **19**) with a benzoyl or a cinnamoyl group at C-1 increased the activity against HL60 cells. On the contrary to this, acetylation at C-3 reduced activity against HL60, AZ521, and SK-BR-3 cells for salannins possessing a tigloyl group at C-1 (**12** *vs*. **13**), and 1-*O*-detigloyl-1-*O*-benzoylsalannins (**20** *vs*. **19**) against AZ521 and SK-BR-3 cells.

(iii) Substitution of a methacrylic group at C-1 with a benzoyl group reduced the activity against HL60 and AZ521 cells for 28-oxo-salannin-type limonoids (**31** vs. **30**/**29**).

(iv) Substitution of a benzoyl or cinnamoyl group at C-1 by a methacrylic or a tigloyl group for nimbin-type limonoids reduced the activity against HL60 cells (**36**, **38** and **39** *vs*. **35** and **37**) in consistent with our recent observation [20, 21].

Table 8.

Cytotoxicity of Extracts and Fractions of *Melia azedarach* against Four Human Cancer Cell Lines

Extract and fraction	IC_{50} (μg/ml)[a])			
	HL 60 (Leukemia)	A549 (Lung)	AZ521 (Duodenum)	SK-BR-3 (Breast)
Fruits				
n-Hexane extract	75.5 ± 1.7	>100	77.0 ± 1.1	>100
Defatted *n*-Hexane fraction	2.9 ± 2.4	12.0 ± 0.7	12.3 ± 0.9	21.9 ± 0.6
MeOH extract	2.9 ± 0.4	64.1 ± 5.4	2.9 ± 1.3	21.0 ± 3.3
EtOAc-soluble fraction	0.05[b] ± 0.006	4.2 ± 0.5	1.9 ± 0.2	34.7 ± 2.4
n-BuOH-soluble fraction	1.9 ± 0.4	>100	2.0 ± 0.3	>100
H_2O-soluble fraction	>100 ±	>100	>100	>100
Leaves				
MeOH Extract	7.5 ± 1.0	>100	13.6 ± 2.1	13.4 ± 0.6
EtOAc-Soluble fraction	9.3 ± 0.5	>100	26.2 ± 3.3	21.1 ± 3.1
H_2O-Soluble fraction	91.5 ± 3.9	>100	>100	>100
Bark				
MeOH Extract	48.4 ± 2.1	>100	81.6 ± 1.8	37.7 ± 5.5
EtOAc-Soluble fraction	23.1 ± 1.7	>100	>100	>100
H_2O-Soluble fraction	>100	>100	>100	>100
Cisplatin[c])	1.3 ± 0.3	>100	2.9 ± 0.2	5.6 ± 0.2

[a])IC_{50} Values based on quintuple points. Cells were treated with test samples (1×10^{-4}–1×10^{-6} g/ml) for 48 h, and cell viability was analyzed by the MTT assay. Each value represents the mean ± S.D. (n = 3). [b])Range of concentration of test sample assayed: 1 × 10-6–1 × 10-8 g/ml. [c]) Reference compound.

Table 9
Cytotoxicity of Compounds (**1-53)** against Four Human Cancer Cell Lines.

NO.	Compound	IC_{50} (μM)[a])			
		HL 60 (Leukemia)	A549 (Lung)	AZ521 (Duodenum)	SK-BR-3 (Breast)
1	12-*O*-acetylazedarachin B	0.016	19.0	0.035	>100
2	Meliarachin C	0.65[b]) ± 0.12	63.6 ± 3.5	1.5 ± 0.5	90.4 ± 1.3
3	Toosendanin	0.005[c]) ± 0.0002	>100	0.009[c]) ± 0.001	>100
4	Meliarachin K	91.4 ± 4.0	>100	>100	>100
5	Meliarachin G	82.0 ± 1.9	>100	>100	>100
6	12-Dehydroneoazedarachin D	11.8 ± 1.5	>100	11.8 ± 7.8	>100
7	12-Dehydro-29-exo-neoazedarachin D	9.1 ± 1.0	>100	18.8 ± 8.8	>100
8	Trichilinin D	61.2 ± 7.8	>100	>100	41.5 ± 3.2
9	1-*O*-Cinnamoyltrichilinin	5.8 ± 0.8	>100	16.2 ± 1.7	>100
10	Trichilinin B	22.2 ± 1.0	>100	58.2 ± 5.6	45.1 ± 3.3
11	3-Deacetyl-1,6-diacetylsendanal	3.6 ± 1.0	>100	22.0 ± 1.5	>100
12	Salannin	67.1 ± 4.1	>100	55.4 ± 6.1	88.1 ± 1.2
13	3-*O*-Deacetylsalannin	23.5 ± 8.5	>100	42.1 ± 4.2	30.6 ± 5.7
14	3-Deacetyl-4'-demethylsalannin	9.6 ± 1.2	>100	47.5 ± 3.6	>100
15	3-Deacetyl-3-*O*-tigloylsalannin	26.6 ± 4.7	>100	85.0 ± 2.6	31.6 ± 0.7
16	Ohchinin	56.3 ± 5.1	>100	>100	>100
17	1-*O*-Decinnamoyl-1-*O*-cis-cinnamoylohchinin	32.9 ± 6.7	>100	>100	>100
18	Ohchinin acetate	9.9 ± 0.6	94.3 ± 1.1	>100	35.2 ± 3.6
19	1-*O*-Decinnamoyl-1-*O*-benzoylohchinin	54.8 ± 2.2	82.3 ± 0.3	35.1 ± 3.9	14.9 ± 2.1
20	1-*O*-Decinnamoyl-1-*O*-benzoylohchinin acetate	12.4 ± 2.6	54.5 ± 1.4	59.8 ± 3.5	82.7 ± 0.5
21	Ohchininolide	31.7 ± 4.1	>100	82.9 ± 1.2	>100
22	1-*O*-Decinnamoyl-1-*O*-benzoylohchininolide	14.1 ± 0.3	>100	34.7 ± 5.8	54.5 ± 4.1
23	23-Methoxyohchininolide A	4.9 ± 0.5	>100	>100	>100
24	23-Methoxyohchininolide B	15.2 ± 2.2	>100	30.0 ± 6.3	>100
25	23-Hydroxyohchininolide	25.1 ± 3.2	>100	78.5 ± 2.9	>100
26	1-*O*-Decinnamoyl-1-*O*-benzoyl-23-hydroxyohchininolide	12.6 ± 4.1	90.1 ± 0.2	55.7 ± 7.1	4.3 ± 1.5
27	21-Hydroxyisoohchininolide	22.7 ± 1.6	>100	>100	91.5 ± 2.2
28	17-Defurano-17-oxoohchinin	50.4 ± 8.9	>100	>100	>100
29	3-Deacetyl-28-oxosalannin	39.1 ± 3.6	>100	>100	>100
30	1-*O*-Decinnamoyl-1-*O*-benzoyl-28-oxoohchinin	22.7 ± 2.4	>100	61.7 ± 7.1	>100
31	3-Deacetyl-4'-demethyl-28-oxosalannin	2.8 ± 0.6	>100	3.2 ± 0.6	>100
32	3-Deacetyl-28-oxosalannolactone	>100	>100	>100	>100
33	3-Deacetyl-28-oxoisosalanninolide	>100	>100	>100	>100
34	3-Deacetyl-7-defurano-17,28-dioxosalannin	>100	>100	>100	>100
35	Ohchinolal	39.2 ± 4.4	>100	>100	94.4 ± 1.0
36	1-Detigloylohchinolal	5.0 ± 0.9	25.7 ± 2.5	7.3 ± 1.3	76.5 ± 5.8
37	Mesendanin E	32.1 ± 3.7	>100	80.0 ± 0.8	70.5 ± 3.8
38	1-Detigloyl-1-*O*-benzylohchinolal	5.1 ± 1.3	83.5 ± 2.6	81.6 ± 1.3	85.0 ± 0.8
39	1-Detigloyl-1-cinnamoylohchinolal	8.5 ± 2.8	>100	>100	94.8 ± 1.5
40	Nimbolinin D	5.4 ± 2.8	>100	96.8 ± 0.6	60.8 ± 9.1
41	Meliasenin E	6.7 ± 0.6	42.3 ± 9.6	11.9 ± 0.8	18.6 ± 2.3
42	Methyl kulonate	5.8 ± 0.5	11.0 ± 1.4	18.9 ± 1.5	16.9 ± 3.7
43	Azedarachic acid	4.6 ± 0.6	>100	21.5 ± 3.8	49.7 ± 4.3
44	Loliolide	>100	>100	>100	>100
45	Methyl indole 3-carboxylate	>100	>100	>100	>100
46	2α,3α,20R-Trihydroxy-5α-pregnane 16β-methacrylate	>100	59.3 ± 7.4	42.5 ± 6.3	26.0 ± 3.6
47	Sitosterol	77.1 ± 2.3	>100	>100	>100
48	2,19-Oxymeliavosin	>100	>100	>100	>100
49	Toosendansterol A	>100	>100	>100	>100
50	5,6-Dehydrotoosendansterol A	>100	>100	>100	>100
51	3-*O*-β-D-glucopyranoside	>100	>100	>100	>100
52	(+)-Catechin	>100	>100	>100	>100
53	(-)-Epicatechin	>100	>100	>100	>100
	Cisplatin[d])	4.2 ± 1.1	18.4 ± 1.9	9.5 ± 0.5	18.8 ± 0.6
	5-Fluorouracil[d])	6.3 ± 2.3	>100	28.7 ± 0.5	>100

[a]) IC_{50} Values based on quintuple points. Cells were treated with compounds (1×10^{-4}–1×10^{-6} M) for 48 h, and cell viability was analyzed by the MTT assay. Each value represents the mean ± S.D. (n = 3). [b]) Range of concentration of test compound assayed: 3×10-6 –3×10-8 M. [c]) Range of concentration of test compound assayed: 3×10-7–3×10-9 M. [d]) Reference compound.

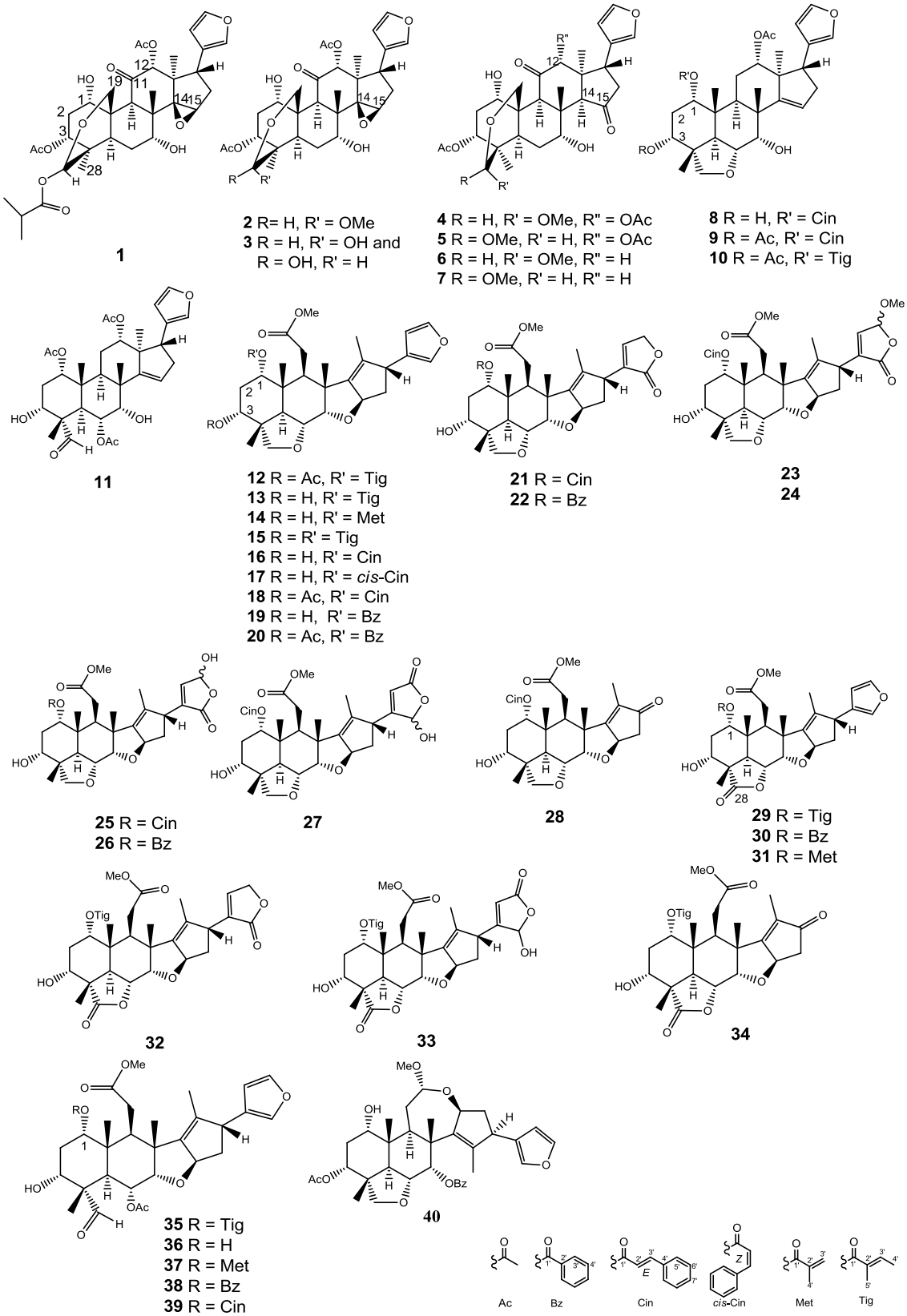

Figure 6. Structures of limonoids (1-40)

4.3 Inhibition of LPS-Induced NO-Release

Macrophages are the major type of cells that participate in inflammation process by producing cytokines/chemokines and various inflammatory mediators such as nitric oxide (NO) and prostaglandins (PGs) [63-65]. Inducible NO synthase (iNOS) is the enzyme that synthesizes NO from L-arginine using NADPH and oxygen molecules [71, 72]. The inhibitory activity toward NO production, induced by lipopolysaccharides (LPS), by murine macrophage-derived RAW 264.7 cells was assayed. Table 11 shows the results as to the NO-production inhibitory and cytotoxic activities against RAW264.7 cells of thirteen compounds (**10**, **16**, **25**, **26**, **29**, **31**-**34**, **42**, **44**-**46**) including nine limonoids among which seven limonoids (**25**-**26**, **29**, **31**-**34**) were new compounds, along with those of the positive control, N5-(Methylamidino)-L-ornithine acetate salt (L-NMMA), an iNOS inhibitor were collected [73]. Of the compounds tested, an euphane-type triterpenoid (**42**) (IC_{50} 4.6 μM) followed by salannin-type limonoid (**16**) (IC_{50} 29.7 μM), exhibited potent inhibitory activities without cytotoxicity (IC_{50} >100 μM) which were more potent than that of L-NMMA (NO-production inhibitory activity, IC_{50} 65.6 μM; cytotoxicity, IC_{50} >100 μM).

In addition, four salannin-type limonoids (**10**, **25**, **26**, **32**), among which liomonoid (**32**) was a new compound, and an indole alkaloid (**45**) also exhibited inhibitory activities (IC_{50} 24.2–87.3 μM), with no (**25**, **26**, **32**, **45**: IC_{50} >100 μM) or weak (**10**: IC_{50} 93.2 μM) toxicity to the cells, which were more potent than, or almost comparable with, that of L-NMMA. Compound (**46**) also showed inhibitory activity (IC_{50} 57.8 μM), but this was cytotoxic to the cells to some extent (IC_{50} 58.3 μM). On the basis of the results in Tables 10 and 11, it can be concluded that compounds (**10**, **16**, **25**, **26**, **32**, **45**) are, at least in part, responsible for the NO-production inhibitory activities of the EtOAc-soluble fraction of the MeOH extract of *M. azedarach* leaves against RAW 264.7 cells. Therefore, it appears that compounds (**10**, **16**, **25**, **26**, **32**, **45**) may be valuable as potential anti-inflammatory bioactivity. It might be worthy to note that

several vilasinin- and salannin-type limonoids [74], in addition to azadirone-, gedunin-, and nimbin-type limonoids [75], isolated from the seeds of *Azadirachta indica*, have been proved to possess potent inhibitory activities against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice.

Table 10.

NO-Release Inhibitory Activities in RAW 264.7 Cells of Compounds Isolated from *Melia azedarach* Extracts

No.	Compound	NO Production-inhibitory activity, IC_{50} [μM][b])	Cytotoxicity, IC_{50} [μM][a]) RAW 264.7
	Vilasinin-type limonoid		
10	Trichilinin B	24.2 ± 7.2	93.2 ± 4.3
	Salannin-type limonoid		
16	Ohchinin	29.7 ± 1.2	>100
25	23-Hydroxyohchininolide	58.6 ± 1.9	>100
26	21-Hydroxyisoohchininolide	87.3 ± 4.5	>100
29	3-Deacetyl-28-oxosalannin	>100	>100
31	3-Deacetyl-4'-demethyl-28-oxosalannin	>100	9.0 ± 0.8
32	3-Deacetyl-28-oxosalannolactone	86.0 ± 4.2	>100
33	3-Deacetyl-28-oxoisosalanninolide	>100	>100
34	3-Deacetyl-7-defurano-17,28-dioxosalannin	>100	>100
	Triterpenoid		
42	Methyl kulonate	4.6 ± 0.1	>100
	Apocarotenoid		
44	Loliolide	>100	>100
	Indole alkaloid		
45	Methyl indole 3-carboxylate	42.2 ± 2.7	>100
	Steroid		
46	2α,3α,20*R*-Trihydroxy-5α-pregnane 16β-methacrylate	57.8 ± 3.4	58.3 ± 0.4
	L-NMMA[c])	65.6 ± 1.9	>100

[a]) IC_{50} values based on quintuple points. Cells were treated with test samples (1×10^{-4}–1×10^{-6} M) for 48 h, and cell viability was analyzed by the MTT assay. Each value represents the mean ± S.D. (n = 3). [b]) IC_{50} values based on quituple points. RAW 264.7 cells were treated with LPS alone or together with the test samples. After 16 h incubation, the supernatants were treated by Griess reagent and the inhibitory rates were calculated. Each value represents the mean ± S.D. (n = 3). [c]) Reference compounds.

Table 11.

NO-Release Inhibitory Activities in RAW 264.7 Cells of *Melia azedarach* Extracts

Extract or fraction	NO Production-inhibitory activity, IC_{50} [μg/ml][b])	Cytotoxicity, IC_{50} [μg/ml][a])
		RAW 264.7
Leaves		
MeOH Extract	10.1 ± 2.0	70.7 ± 4.2
EtOAc-Soluble fraction	11.9 ± 2.0	77.4 ± 8.1
H_2O-Soluble fraction	>100	>100
Bark		
MeOH Extract	27.0 ± 6.8	95.3 ± 0.7
EtOAc-Soluble fraction	>100	95.1 ± 1.8
H_2O-Soluble fraction	>100	>100
L-NMMA[c])	65.6 ± 1.9	>100

[a]) IC_{50} values based on quintuple points. Cells were treated with test samples (1×10^{-4}–1×10^{-6} g/ml) for 48 h, and cell viability was analyzed by the MTT assay. Each value represents the mean ± S.D. (n = 3). [b]) IC_{50} values based on quituple points. RAW 264.7 cells were treated with lipopolysaccharide (LPS) alone or together with the test samples. After 16 h incubation, the supernatants were treated by Griess reagent and the inhibitory rates were calculated. Each value represents the mean ± S.D. (n = 3). [c]) Reference compound.

4.4 Evaluation of Cell Death Pathway

Three limonoids (**1**, **2**, **31**), among which limonoid (**31**) was a new compound, were evaluated for their apoptosis-inducing activity by flow cytometry of HL60 cells and AZ521 cells stained with annexin V-FITC and PI. Exposure of the membrane phospholipid, phosphatidylserine, to the external cellular environment is one of the earliest markers of apoptotic cell death [76]. Annexin V is a calcium-dependent phospholipid binding protein with high affinity for phosphatidylserine expressed on the cell surface. PI does not enter a cell with intact cell-membrane and was used to differentiate between early apoptotic (annexin V-positive, PI-negative), late apoptotic (annexin V-, PI-double positive), or necrotic cell death (annexin V-negative, PI-positive).

The ratio of early apoptotic cells (lower right) was increased after treatment with limonoid (**1**) in HL60 for 24 h (from 0.5 to 9.4 %) and 48 h (20.0 %), and that of late apoptotic cells (upper right) was increased after 24 h (from 0.1 to 3.0 %) and 48 h (21.3 %) (Figure 7A). The ratio of early apoptotic cells (lower right) was increased after

treatment with limonoid (**2**) in HL60 cells for 24 h (14.9% *vs.* 1.8% of negative control) and 48 h (52.1% *vs.* 2.1% of negative control), and that of late apoptotic cells (upper right) was increased after 48 h (31.6% *vs.* 1.2% of negative control) (Figure 7B). In the case of limonoid (**31**), which is a new compound, the ratio of early apoptotic cells was increased after treatment for 48 h (31.6% *vs*. 2.1% of negative control), and that of late apoptotic cells (upper right) was increased after 24 h (38.7% *vs*. 1.2% of negative control) (Figure 7C). These results revealed that most of the cytotoxic activities of three limonoids (**1**, **2**, **31**) against HL60 cells are due to inducing apoptotic cell death.

Two limonoids (**1**, **31**) were also evaluated for apoptosis-inducing activity against AZ521 cells by means of annexin V and PI double staining. The ratio of early apoptotic cells was increased with limonoid (**1**), but only a slight extent, after treatment with compound (50 nM) for 24 h (from 2.6 to 3.1 %) and for 48 h (7.6 %), and that of late apoptotic cells was increased significantly for 48 h (from 5.2 to 58.0 %) (Figure 7D). The ratio of necrotic cells (upper left) was, however, also increased after treatment with the compound for 48 h (from 3.7 to 18.9 %). The other ratio of early apoptotic cells was increased with the new compound (**31**) for 24 h (from 2.7% to 28.8%) and 48 h (16.8%), and that of late apoptotic cells (upper right) increased after 48 h (from 1.1% to 41.0%) (Figure 7E). These results suggested that cytotoxicity of limonoids (**1**) and new limonoid (**31**) against AZ521 cells is due to inducing apoptotic cell death as well as necrotic cell death with the latter predominated.

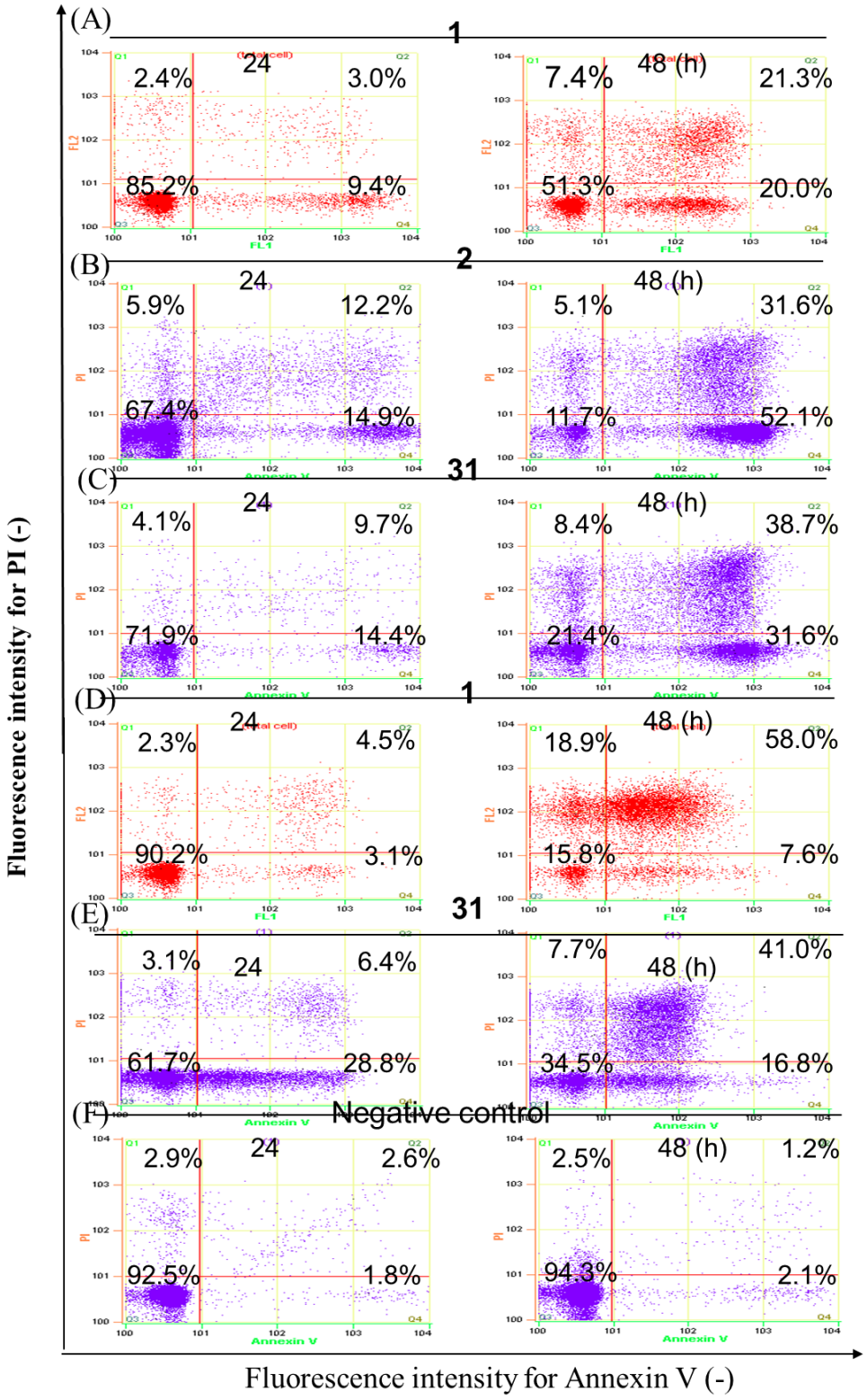
(A)
1
24
2.4%
3.0%
85.2%
9.4%
48 (h)
7.4%
21.3%
51.3%
20.0%
(B)
2
24
5.9%
12.2%
67.4%
14.9%
48 (h)
5.1%
31.6%
11.7%
52.1%
(C)
31
24
4.1%
9.7%
71.9%
14.4%
48 (h)
8.4%
38.7%
21.4%
31.6%
(D)
1
24
2.3%
4.5%
90.2%
3.1%
48 (h)
18.9%
58.0%
15.8%
7.6%
(E)
31
24
3.1%
6.4%
61.7%
28.8%
48 (h)
7.7%
41.0%
34.5%
16.8%
(F)
Negative control
24
2.9%
2.6%
92.5%
1.8%
48 (h)
2.5%
1.2%
94.3%
2.1%
Fluorescence intensity for PI (-)
Fluorescence intensity for Annexin V (-)

Figure 7. Detection of compounds (**1**, **2**, **31**) induced apoptosis against HL60 cells and AZ521 cells

(A) HL60 cells were cultured with compound (**1**) (5 μM) for 24 and 48h. (B) HL60 cells were cultured with compound (**2**) (5 μM) for 24 and 48h. (C) HL60 cells were cultured with compound (**31**) (10 μM) for 24 and 48h. (D) AZ521 cells were cultured with compound (**1)** (5 μM) for 24 and 48h. (E) AZ521 cells were cultured with compound (**31**) (10 μM) for 24 and 48h. (F) Negative control. Each value is the mean of three experiment.

4.5 Analysis of Apoptosis Induction Mechanism

Caspases are known to mediate the apoptotic pathway [77, 78]. Caspases-8 and -9, which are initiator caspases, appear to be the apical caspases activated in death receptor- and mitochondrial stress-induced apoptotic cell death, respectively. Initiator caspases are responsible for either directly or indirectly activating various effector caspases, including caspases-3, -6, and -7, which contain short prodomains. Effector caspases cleave a number of structural and regulatory proteins and are directly responsible for many of the apoptotic features, which are nuclear and cytoplasmic condensation, DNA fragmentation, cell membrane composition, and others [79]. In order to clarify the mechanisms by which three limonoids (**1**, **2**, **31**) induce apoptotic cell death, activation of caspases-3, -8, and -9 was evaluated by Western blot analysis. The levels of procaspases-8, -9, and -3 diminished, almost in a time-dependent manner, after treatment with limonoids (**2**, **31**) (Figure 8). These results suggest that limonoids (**1**, **2**, **31**), among which limonoid (**31**) was new compound, induced apoptotic cell death *via* both the mitochondrial- and the death receptor mediated pathways.

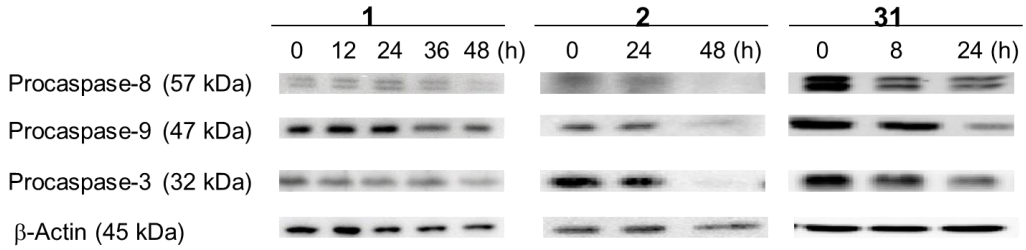

Figure 8. Western blot analysis of HL-60 cell treated with compound (**1**) (5 μM), (**2**) (5 μM), and (**31**) (10 μM). The results are from one representative experiment among three runs, which showed similar patterns to one another.

4.6 Analysis of NO-Release Inhibitory Activity by Western Blot

There are two isoforms of cyclooxygenase (COX), designated COX-1 and COX-2, which catalyze the biosynthesis of PGs from arachidonic acid [72]. COX-1 is constitutively expressed in most tissues and seems to be responsible for housekeeping roles for normal physiological functions. In contrast, COX-2 is not detectable in most normal tissues, but is induced by bacterial LPS, TPA, or cytokines in macrophages, fibroblasts, and inflamed tissues [72, 80]. Since COX-2 and iNOS are mainly responsible for the production of large amounts of PGs and NO, respectively, the potent NO-production inhibitor, limonoid (**16**), found in this study, was evaluated for its inhibitory effects on the expression of iNOS and COX-2 as well as COX-1 in LPS-stimulated RAW 264.7 macrophages by Western blotting. As shown in Figure 9, treatment of LPS-induced macrophage cells with limonoid (**16**) significantly suppressed the expression of iNOS and COX-2 proteins in concentration-dependent manner while unaffected the expression of COX-1 protein. These findings suggest that the reduction in the expression of iNOS protein contributed to the inhibitory activity of limonoid (**16**) on LPS-induced NO-production. In addition, limonoid (**16**) would be a potent suppressor of PGs production by inhibiting the expression of COX-2 selectively.

Evaluation of another potent NO-production inhibitor, limonoid (**42**), on the expression of iNOS, COX-1, and COX-2 proteins is under investigation.

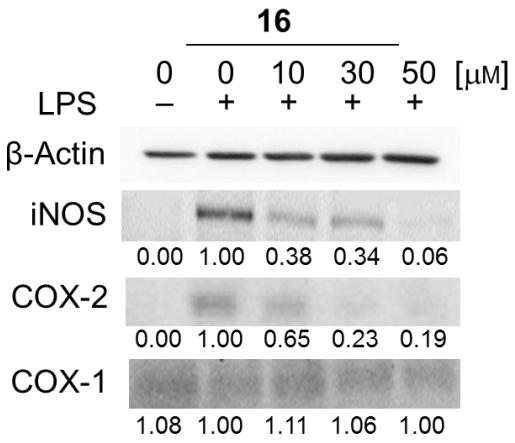

Figure 9. Inhibitory effects of compound (**16**) on the expression of iNOS, COX-2, and COX-1 proteins induced by LPS in RAW 264.7 cells. Relative amount of expressed protein is shown at each immunoblotting band. The results are from one representative experiment among three runs, which showed similar patterns to one another.

4.7 Selectivity of Cytotoxicity between Normal Lymphocyte Cells and Cancer Cell Lines

Limonoid (**2**) and new limonid (**31**) and cisplatin were then tested for their cytotoxicity against a normal lymphocyte cell line, RPMI 1788, and their selectivity index (SI) value [81], which was determined by dividing the IC_{50} value for the normal cell line (RPMI 1788) by the IC50 value for the cancer cell line (HL60). As shown in Table 12, Limonoid (**2**) and new limonid (**31**) exhibited SI values of 16.31 and 5.36, respectively, greater than that of cisplatin (SI 0.33).

From the results of the cytotoxicity evaluation of the limonoids from *Melia azedarach* fruit extracts, it appears that some of these compounds may be valuable

anticancer lead compounds. Furthermore, limonoid (**2**) and new limonid (**31**) which induced apoptotic cell death in leukemia and displayed high selective toxicity against leukemia, may be especially promising in this regard. The cytotoxicity of compound (**3**) against U937 (histocyte) cells has previously been revealed to be associated with the induction of apoptosis [68].

Table12.

Cytotoxic activities of compounds **2** and **31** and cisplatin against leukemia (HL60) and normal lymphocyte (RPMI 1788) cell lines.

Compound	IC_{50} (μM)[a])		SI[b])
	HL60 (Leukemia)	RPMI 1788 (Normal lymphocyte)	
2	0.65 ± 0.12	10.6 ± 3.1	16.31
31	2.8 ± 0.6	15.0 ± 8.9	5.36
Cisplatin[c])	4.2 ± 1.1	1.4 ± 0.03	0.33

[a]) IC_{50} Values based on quintuple points. Cells were treated with compounds (1×10^{-4}–1×10^{-8} M) for 48 h, and cell viability was analyzed by the MTT assay. Each value represents the mean ± S.D. (n = 3). [b])SI refers to the selectivity index, which was obtained by dividing the IC50 value for the normal lymphocyte cells by the IC50 value for HL60 cells. c) Reference compound.

Chapter 5 Conclusion

The constituents from *M. azedarach* of the MeOH extract and the hexane extract of fruits, the MeOH extracts of leaves and the MeOH extracts of bark fruits, has been elucidated in this study.

From the MeOH extract of the *M. azedarach* fruits, thirty three compounds including thirty two limonoids (**1**-**9**, **12**-**13**, **15**-**28**, **30**-**31**, **35**, **37**-**40**), and one triterpenoid (**41**) were isolated. Among these thirty three compounds, fourteen limonoids (**6**, **7**, **17**, **19**, **21**-**28**, **30**, **31**) were confirmed as new compounds.

Twenty compounds, including nineteen limonoids (**8**-**9**, **11**, **13**-**14**, **16**, **18**, **21**-**25**, **29**-**31**, **35**, **36**) and one triterpenoid (**43**) were isolated from the hexane extract of *M. azedarach* fruits. Among these twenty compounds, three limonoids (**11**, **14**, **29**) were confirmed as new compounds.

From the MeOH extract of *M. azedarach* leaves and bark, nine limonoids (**10**, **16**, **25**, **27**, **29**, **31**-**34**), one triterpenoid (**42**), one apocarotenoid (**44**), one alkaloid (**45**), six steroids (**46**-**51**), two flavonoids (**52**-**53**), including three new limonoids (**32**, **33**, **34**) were isolated.

In order to evaluate biological activities of the compounds (**1**-**53**) isolated from the *M. azedarach*, inhibitory effects on inhibition of TPA induced EVB-EA activation, cytotoxicity against four human cancer cell lines, and NO-release inhibition were evaluated.

The inhibitory effects on the induction of EBV-EA induced by TPA of thirty nine compounds (**2**-**9**, **16**-**17**, **19**-**35**, **37**, **40**-**41**, **44**, **46**-**53**) including twenty nine limonoids, among which eighteen limonoids (**6**, **7**, **17**, **19**, **21**-**34**) were new compounds, were examined for preliminary evaluation of anti-tumor promoting activity. All of the thirty nine compounds tested exhibited moderate inhibitory effects on preservation of high viability of the Raji cells. Among tested thirty nine compounds, nine compounds (**2**, **7**,

33, **34**, **37**, **46**, **51-53**), including five limonoids (**2**, **7**, **33**, **34**, **37**), among which three limonoids (**7**, **33**, **34**) were new compounds, exhibited more potent than against TPA induced EBV-EA activation in Raji cells. Since the inhibitory effects against EBV-EA activation have been demonstrated to closely parallel to inhibition of tumor promotion *in vivo* [19], it was deduced that the nine compounds (**2**, **7**, **33**, **34**, **37**, **46**, **51-53**) are potential inhibitors of tumor promotion.

Furthermore, by evaluation of fifty three compounds (**1-53**) including forty limonoids, among which twenty limonoids (**6**, **7**, **14**, **17**, **19**, **21-34**, **36**) were new compounds, for their cytotoxic activities against four human cancer cell lines (HL60, A549, AZ521, and SK-BR-3), it was found that, three limonoids (**1**, **2**, **31**), among which limonoid (**31**) was a new compound, exhibited potent cytotoxic activity against HL60 cell lines and AZ521 cell lines. In addition, limonoids (**1**, **2**, **31**) were demonstrated mainly due to induction of apoptosis by flow cytometry. Result of western blot analysis suggested that **1**, **2**, **31** induced apoptosis *via* both the mitochondrial and death receptor-mediated pathways in HL60 cells, maybe promising lead compounds for developing effective drug for leukemia.

Next, thirteen compounds (**10**, **16**, **25**, **26**, **29**, **31-34**, **42**, **44-46**) including nine limonoids, among which seven limonoids (**25-26**, **29**, **31-34**) were new compounds, were tested inhibitory activity against LPS-induced NO-release in RAW 264.7 cells. Among thirteen compounds tested, seven compounds (**10**, **16**, **25**, **26**, **32**, **42**, **45**) exhibited inhibitory activities with no, or almost no, cytotoxicity, and limonoid (**16**) and compound (**42**) exhibited potent NO-release inhibitory activities. From the results of western blot analysis, limonoid (**16**) reduced the expression levels of iNOS and COX-2 in a concentration dependent manner suggesting that the inhibitory activity of limonoid (**16**) on LPS-induced NO-release reduction is due to inhibiting the expression of iNOS. From above mentioned resulted, it was deduced that, seven compounds (**10**, **16**, **25**, **26**, **32**, **42**, **45**) may be valuable as candidates of anti-inflammatory agents.

This study has suggested that constituents including limonoids from various parts of

M. azedarach have possibility to be used as material of innovative drug developments. Thus, perhaps remodeling of components for enhances of bioactivities and depression of side effects could bring us a step closer to the commercialization of natural products against all of lifestyle-related diseases.

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Appendix

1. List of Compounds Appeared in This Dissertation

Number	Compound
1	12-*O* -Acetylazedarachin B
2	Meliarachin C
3	Toosendanin
4	Meliarachin K
5	Meliarachin G
6	12-Dehydroneoazedarachin D
7	12-Dehydro-29-exo-neoazedarachin D
8	Trichilinin D
9	1-*O* -Cinnamoyltrichilinin
10	Trichilinin B
11	3-Deacetyl-1,6-diacetylsendanal
12	Salannin
13	3-*O* -Deacetylsalannin
14	3-Deacetyl-4'-demethylsalannin
15	3-Deacetyl-3-*O* -tigloylsalannin
16	Ohchinin
17	1-*O* -Decinnamoyl-1-*O* -cis-cinnamoylohchinin
18	Ohchinin acetate
19	1-*O* -Decinnamoyl-1-*O* -benzoylohchinin
20	1-*O* -Decinnamoyl-1-*O* -benzoylohchinin acetate
21	Ohchininolide
22	1-*O* -Decinnamoyl-1-*O* -benzoylohchininolide
23	23-Methoxyohchininolide A
24	23-Methoxyohchininolide B
25	23-Hydroxyohchininolide
26	1-*O* -Decinnamoyl-1-*O* -benzoyl-23-hydroxyohchininolide
27	21-Hydroxyisoohchininolide
28	17-Defurano-17-oxoohchinin
29	3-Deacetyl-28-oxosalannin
30	1-*O* -decinnamoyl-1-*O* -benzoyl-28-oxoohchinin
31	3-Deacetyl-4'-demethyl-28-oxosalannin
32	3-Deacetyl-28-oxosalannolactone
33	3-Deacetyl-28-oxoisosalanninolide
34	3-Deacetyl-7-defurano-17,28-dioxosalannin
35	Ohchinolal
36	1-Detigloylohchinolal
37	Mesendanin E
38	1-Detigloyl-1-*O* -benzylohchinolal
39	1-Detigloyl-1-cinnamoylohchinolal
40	Nimbolinin D
41	Meliasenin E
42	Methyl kulonate
43	Azedarachic acid
44	Loliolide
45	Methyl indole 3-carboxylate
46	2α,3α,20R-Trihydroxy-5α-pregnane 16β-methacrylate
47	Sitosterol
48	2,19-Oxymeliavosin
49	Toosendansterol A
50	5,6-Dehydrotoosendansterol A
51	3-*O* -β-D-Glucopyranoside
52	(+)-Catechin
53	(-)-Epicatechin

2. List of Abbreviations

AcOH	Acetic acid
n-BuOH	*n*-Butanol
COSY	Correlated spectroscopy
COX	Cyclooxygenase
DEPT	Distortionless enhancement by polarization transfer
DMSO	Dimethylsulfoxide
EBV-EA	Epstein-Barr virus early antigen
ECL	Enhanced chemiluminescence
ED_{50}	50% Effective dose
EtOAc	Ethyl acetate
EtOH	Ethanol
FITC	Fluorescein isothiocyanate
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum coherence
HPLC	High-performance liquid chromatography
HRESIMS	High-resolution electrospray ionisation mass spectrometry
HRMS	High-resolution mass spectrometry
IC_{50}	50% Inhibitory concentration
IR	Infrared spectroscopy
MeCN	Acetonitrile
M.p.	Melting point
MeOH	Methanol
NO	Nitric oxide
iNOS	Nitric oxide Synthase
NOE	Nuclear overhauser effect
NOESY	Nuclear overhauser enhancement and exchange spectrooscopy
NMR	Nuclear magnetic resonance
L-NMMA	N^G-monomethyl-L-arginine
ODS	Octadecyl silica
PBS	Phosphate buffered saline
PGs	Prostaglandins
PI	Propidium iodide
TPA	12-*O*-Tetradecanoylphorbol-13-acetate
t_R	Retention time
VDAC	Voltage dependent anion channel